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### CORRECTIONS.

On page 242, Vol. lxxi, No. 2, January, 1927, Table I, the factors for 28 and 29°C. should read:

28	3104 instead of 3004
29	3092 <span style="float: right;">92</span>

### CORRECTIONS.

On page 146, Vol. lxxvii, No. 1, January, 1926, line 3, Equation 2 should read

$$\frac{\text{CO}_2 - \left[ \frac{1.404 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1) + 1} \times 0.713 \right]}{2.24} = B \text{ as } \text{BHCO}_3$$

instead of

$$\frac{\text{CO}_2 - \left[ \frac{14.04 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1)} \times 0.713 \right]}{2.24} = B \text{ as } \text{BHCO}_3$$

and the fraction in line 4 should read

$$\frac{1.404 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1) + 1} \text{ instead of } \frac{14.04 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1)}$$

The calculations in the text were derived from the correct equation.





## THE CATALYTIC FORMATION OF MIXED CHOLESTERYL ETHERS.

By CHARLES E. BILLS AND FRANCIS G. McDONALD.

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.)

(Received for publication, November 22, 1926.)

In our studies on the catalytic alteration of sterols (1, 2) it was demonstrated that cholesterol yields upon moderate treatment with floridin a crystalline body of high molecular weight. From certain experimental data we were led to suggest tentatively (2) that the derivative might be a polymer of cholesterol. This view we are compelled to abandon in the light of further research.

We reported that this substance, like cholesterol itself, reacts with Hübl's solution in so erratic a manner that reliable determinations of the Hübl iodine value are impossible. However, we now find, in accord with the experience of Dam (3, 4), that the Rosenmund-Kuhnhehn reagent (5) affords a means of determining the iodine numbers of sterols with considerable precision. Purified cholesterol gave the value 68.6 (theoretical, 65.7); the crystalline substance, 69.1. It was difficult to conceive how, after polymerization, the halogen absorption could be as great, or greater, than before.

We thus came to reexamine our previous data. The found molecular weight, 978, had been determined by the new method of Rast (6), which consists in measuring the melting point depression of the solution in *d*-camphor by means of an ordinary thermometer and capillary. Now, since the depression is 40° per mol, it may be computed that for a 6 per cent solution, such as we used, a substance having a molecular weight of, say, 800, would indicate a weight of 1200 if the reading of the thermometer were 1° low. Realizing this, we have repeatedly practiced recognizing the difficult exact end-point of the melting camphor solution. Of late we have been able to obtain fairly concordant readings

which indicate that the molecular weight of the crystalline substance actually lies between 725 and 775.

These limits suggest that the substance may be dicholesteryl ether, formed by the catalytic dehydration of cholesterol (calculated molecular weight = 755; iodine No. = 67.3). However, the percentage composition of dicholesteryl ether is not greatly different from that of the hypothetical dicholesterol, and the single analysis which we reported agrees fairly well with each. Three additional combustions have proved of no greater distinguishing value.

*Percentage Composition.*

	C	H
Theoretical for dicholesteryl ether.....	85.86	12.02
“ “ cholesterol polymers.....	83.86	12.00
Found for 0.2 gm. samples of X.....	II 84.4	12.5
	III 84.1	12.2
	IV 84.6	12.3

It will be noted that the above combustions reflect a somewhat high water absorption, but this is not surprising, since it is known that many sterols are notoriously difficult to free from traces of liquid, solid, or even gaseous impurities. It remains to compare our substance with the previously described ethers of cholesterol.

The purest preparations of our derivative melt to a crystalline liquid at 203°, which becomes clear at 209°, and remelts at 196°. Mauthner and Suida (7) obtained a substance which sintered at 188° and cleared at 195° by heating cholesterol with anhydrous copper sulfate. Their analytical and molecular weight determinations indicated cholesteryl ether. The crystal form and solubilities of their product agree with ours, and it may be added that we have often examined impure preparations of our product which melted at as low as 195°, or even lower. Minovici (8) treated cholesterol with aqueous sulfuric acid, obtaining, among other substances, an ether which softened at 195° and cleared at 201°. Notwithstanding that the constants of these ethers were similar, Minovici suggested that they may be isomeric. Steinkopf and Blümner (9) regarded Minovici's suggested isomerism with disfavor. They prepared an ether "completely identical" with Mauthner and Suida's by the action of picryl chloride on potassium cholesterylolate. Steinkopf, Winternitz, Roederer, and Wolynski (10) obtained an ether melting at 194.5° by fusing cholesterol with kieselguhr.<sup>1</sup>

<sup>1</sup> Incorrectly translated "fuller's earth" in *Chem. Abst.*, 1920, xiv, 3635.

Our product is formed not only by floridin, but by a variety of dehydrating catalysts, including kieselguhr. The breadth of this reaction is demonstrated as follows: 1 gm. of cholesterol in 30 cc. of carbon tetrachloride is refluxed with 5 gm. of activated kieselguhr, or other earthy catalyst, instead of floridin. The optimum period of refluxing varies with the activity of the catalyst. Floridin, the most active (and destructive) of all, requires but a few minutes; kieselguhr, 2 hours, and a few earths several hours. The reaction mixture is extracted and purified as previously described (2). In this way we have obtained good yields with kieselguhr, kaolin, bentonite (two samples), bauxite, and various fuller's earths (eighteen samples from the principal deposits in the United States and England). Even talc exhibits a slight action, but pumice, silica gel, natural amorphous silica, permutit, powdered glass, bone-black, and vegetable charcoal are indifferent. Fuller's earths from different localities were found to be exceedingly variable in activity, so much so that we suggest that in biochemical literature fuller's earth signifies little, unless the origin is specified.

While there remained little doubt that our crystalline substance was dicholesteryl ether, final proof was afforded by the synthesis and comparison of a series of mixed ethers. It was ascertained that when the catalysis of cholesterol is conducted in a benzene hydrocarbon,<sup>2</sup> the presence of another alcohol or phenol, in a quantity peculiarly suited to each case, occasions the formation of the corresponding mixed cholesteryl ether. This simple reaction is widely applicable. For example, from *d*-borneol, which, like cholesterol, is a secondary terpene alcohol, we made cholesteryl *d*-bornyl ether; from phenol, cholesteryl phenyl ether; and from several aliphatic alcohols, the respective mixed ethers. Even a somewhat labile unsaturated alcohol such as allyl alcohol lends itself to the reaction fairly well; the highly unstable tertiary amyl alcohol was, however, decomposed.

In the following experimental descriptions few general rules can be laid down. The apparatus consists of a 500 cc. Kjeldahl flask and reflux condenser. The cholesterol and its solvent, with the special alcohol, are mixed first, and the floridin added to this

<sup>2</sup> The benzene hydrocarbons are more suitable than carbon tetrachloride, for the reason that the latter reacts slightly with some of the alcohols.

solution. The floridin<sup>3</sup> is activated beforehand by heating for 2 hours at 300°. As the reaction proceeds the floridin suspension becomes slightly darker in color, more so in some preparations than in others. The duration of treatment is determined by the solvent employed (benzene, toluene, or xylene), the nature and quantity of the special alcohol, and the activity of the catalyst. When the catalysis is complete the action is promptly terminated by chilling the flask. The mixture is then filtered with suction, and the floridin residue extracted several times with ether. The combined filtrate and lixivium are evaporated to dryness over steam, giving what is referred to below as the "crude product." This should be a more or less yellow mass of waxy consistency, which in most cases melts on moderate warming.

Purification of the crude product is accomplished by repeated crystallization. The best crystals are usually obtained by chilling hot, saturated solutions for several hours in the ice box. The *d*-bornyl, phenyl, and isopropyl cholesteryl ethers are easily purified because of their insolubility in alcohol. In all other cases it is well to test the first crop of crystals for the presence of free cholesterol. This is done either by the digitonin reaction or by means of a modified Moleschott reagent which we devised for this purpose. The reagent is simply 10 M H<sub>2</sub>SO<sub>4</sub>, prepared by diluting 56 cc. of sulfuric acid, sp. gr. 1.84, to 100 cc. A particle of the crystallized ether is added to a few cc. of the reagent in a test-tube, and the tube immersed in boiling water for 2 to 3 minutes. The pure ethers remain unchanged; free cholesterol becomes red. It is practically impossible to purify a preparation containing more than a trace of cholesterol. It is only less difficult to isolate the ether from mixtures containing large amounts of the resinous decomposition products of unduly prolonged actions. The alcohol-insoluble ethers are crystallized from a mixture of ether and alcohol; the others from alcohol or acetone. Acetone generally gives the finest crystals, but alcohol the greatest yield. When the low melting ethers are added to the hot solvent they may melt to an oily globule requiring prolonged digestion for complete solution. If a persistently insoluble, *solid* residue,

<sup>3</sup> Kindly furnished, as a "200 mesh" powder, by the Floridin Company of Warren, Pennsylvania, from its mine at Jamieson, Florida.

or flocculent suspension appears in the hot solution, it must be removed (traces of dicholesteryl ether).

*Cholesteryl d-Bornyl Ether*.—Reflux 5.0 gm. of cholesterol, 25 gm. of *d*-borneol, 125 cc. of benzene, and 25 gm. of floridin for 30 minutes. Dissolve the crude product in a minimum of boiling ether, cool, and add cold 95 per cent alcohol with constant shaking until a good precipitation is obtained. Allow to stand several hours in the ice box, filter, wash with alcohol, and recrystallize from ether-alcohol until the melting point is constant at 177°. Yield, 4.5 gm.

*Cholesteryl Phenyl Ether*.—Reflux 5.0 gm. of cholesterol, 25 gm. of phenol, 125 cc. of toluene, and 25 gm. of floridin for 10 minutes. Dissolve the crude product in a minimum of boiling ether, cool, and add cold 95 per cent alcohol with constant shaking until a good precipitation is obtained. Allow to stand several hours in the ice box, filter, wash with alcohol, and recrystallize from ether-alcohol until the melting point is constant at 155°. Yield, 3.2 gm.

*Cholesteryl Allyl Ether*.—Reflux 5.0 gm. of cholesterol, 10 cc. of allyl alcohol, 140 cc. of toluene, and 25 gm. of floridin for 4 hours. Dissolve the crude product in absolute alcohol, decolorize by boiling with norit, filter, and evaporate to dryness. Recrystallize from acetone until the melting point is constant at 78°. Yield, 2.0 gm.

*Cholesteryl Secondary Octyl Ether (Cholesteryl Methyl *n*-Hexyl Carbinol Ether)*.—Reflux 5.0 gm. of cholesterol, 35 cc. of methyl *n*-hexyl carbinol, 115 cc. of xylene, and 25 gm. of floridin for 15 minutes. Purify the crude product by crystallization first from acetone, and subsequent recrystallization from 95 per cent alcohol until the melting point is constant at 89°. Yield, 3.5 gm.

*Cholesteryl *n*-Heptyl Ether*.—Reflux 5.0 gm. of cholesterol, 35 cc. of *n*-heptyl alcohol, 115 cc. of xylene, and 25 gm. of floridin for 15 minutes. Purify the crude product by recrystallization from 95 per cent alcohol, washing each crystal crop with 80 per cent alcohol, until the melting point is constant at 96°. Yield, 3.5 gm.

*Cholesteryl *n*-Hexyl Ether*.—Reflux 5.0 gm. of cholesterol, 35 cc. of *n*-hexyl alcohol, 115 cc. of xylene, and 25 gm. of floridin for 25 minutes. Purify the crude product by recrystallization from acetone until the ether melts constantly at 68° to a turbid liquid becoming clear at 79°. Yield, 3.3 gm.

*Cholesteryl Isoamyl Ether*.—Reflux 5.0 gm. of cholesterol, 35 cc. of isoamyl alcohol, 115 cc. of xylene, and 25 gm. of floridin for 7 hours. Purify the crude product by recrystallization from 95 per cent alcohol, washing each crystal crop with 80 per cent alcohol, until the ether melts constantly at 94° to a turbid liquid becoming clear at 98°. Yield, 3.8° gm.

*Cholesteryl Isobutyl Ether*.—Reflux 5.0 gm. of cholesterol, 25 cc. of isobutyl alcohol, 125 cc. of toluene, and 25 gm. of floridin for 7 hours. Purify the crude product by recrystallization from 95 per cent alcohol, washing

each crystal crop with 80 per cent alcohol, until the melting point is constant at 113°. Yield, 3.1 gm.

*Cholesteryl n-Butyl Ether*.—Reflux 5.0 gm. of cholesterol, 15 cc. of *n*-butyl alcohol, 135 cc. of toluene, and 25 gm. of floridin for 8 hours. Purify the crude product by recrystallization from acetone until the ether melts constantly at 79° to a turbid liquid becoming clear at 86°. Yield, 3.5 gm.

*Cholesteryl Isopropyl Ether*.—Reflux 5.0 gm. of cholesterol, 15 cc. of isopropyl alcohol, 135 cc. of toluene, and 25 gm. of floridin for 13 hours. Dissolve the crude product in a minimum of boiling ether, add warm 95 per cent alcohol until precipitation commences, and allow to crystallize. Recrystallize from ether-alcohol until the melting point is constant at 132°. Yield, 2.5 gm.

*Cholesteryl n-Propyl Ether*.—Reflux 5.0 gm. of cholesterol, 10 cc. of *n*-propyl alcohol, 140 cc. of toluene, and 25 gm. of floridin for 16 hours. Purify the crude product by recrystallization from 95 per cent alcohol, washing each crystal crop with 80 per cent alcohol, until the melting point is constant at 100°. Yield, 3.4 gm.

*Cholesteryl Ethyl Ether*.—Reflux 5.0 gm. of cholesterol, 10 cc. of absolute ethyl alcohol, 140 cc. of xylene, and 25 gm. of floridin for 14 hours. Purify the crude product by crystallization first from 95 per cent alcohol, and subsequent recrystallization from acetone until the melting point is constant at 89°. Yield, 3.0 gm.

*Cholesteryl Methyl Ether*.—Reflux 5.0 gm. of cholesterol, 15 cc. of absolute methyl alcohol, 135 cc. of xylene, and 25 gm. of floridin. The reaction does not take place until some of the methyl alcohol is lost by evaporation through the condenser; when it does occur it may easily be carried to excess. The proper period with our set up is about 50 hours, but in general the period must be determined by tests for the disappearance of cholesterol from the reaction mixture. Purify the crude product by recrystallization from acetone until the melting point is constant at 82°. Yield, 2.5 gm.

Four of the mixed ethers prepared by our reaction may be identified with products already described by other investigators. Cholesteryl phenyl ether was prepared by Steinkopf and Blümner (9) by the interaction of sodium phenolate and cholesteryl chloride. Cholesteryl *n*-propyl, ethyl, and methyl ethers were prepared by Diels and Blumberg (11) by treating cholesteryl chloride with the respective alcohols in the presence of magnesium. All four of these ethers agree fairly well with our products in so far as their physical constants have been compared; *i.e.*, melting point, crystal form, and solubilities.

As an analytical procedure we applied the Rosenmund-Kuhn-henn bromination method of determining iodine numbers. In

most cases the halogen absorptions were in good agreement with the theoretical. Cholesteryl phenyl ether gave a value 11 points high—a figure which may be explained by the well known affinity of the phenyl radical for bromine. Cholesteryl allyl ether, which, on account of its doubly unsaturated structure, should have given an iodine value of 119.0, actually gave a value 7.3 points low. The allyl ether differs in another respect from all the other mixed ethers: On standing it gradually changes into a modification exhibiting a higher melting point. The sample on which the iodine number and optical rotation (Table I) were obtained was a month old; it no longer melted sharply, and the clearing point had risen about 20°. One might attribute this instability to the *additional* double bond characteristic of this derivative. So far as we know, the only other cholesterol derivative which changes spontaneously is the antiricketic modification produced by irradiation.

The mixed ethers of cholesterol form well defined, colorless crystals, which hold most tenaciously small amounts of impurities, such as solvents or free cholesterol. They give no precipitate with digitonin and do not react with acetic anhydride. With slight individual differences, all the ethers give the Burchard-Liebermann and Salkowski color reactions of cholesterol. Certain ethers, especially the isoamyl, become highly electrified when rubbed with a metal spatula. Several ethers exhibit color play on cooling from the molten state. For convenience in comparison we have included in Table I the following properties of the mixed ethers and dicholesteryl ether: melting point phenomena, optical rotation, crystalline form, solubility, and iodine number.

Not only cholesterol, but its esters, are converted into ethers by catalysts of the fuller's earth series. For example, when cholesteryl acetate in carbon tetrachloride is treated with floridin in the usual manner (2), the ester structure is ruptured and dicholesteryl ether is produced. Furthermore, the mixed ethers are formed from dicholesteryl ether when the latter is catalyzed in xylene containing, for example, isoamyl alcohol. The reverse reaction, however, does not occur to any considerable extent, for when the mixed ethers are subjected to catalysis they yield resinous pigments almost as readily as cholesteryl ether itself (2). As we previously reported (1), the catalysis of cholesterol takes



## Cholesteryl Ethers

TABLE I.  
*Showing the Physicochemical Constants of the Ethers of Cholesterol.*

Substance.	Melting point phenomena.	$[\alpha]_D^{20}$ of 2 per cent solution in CCl <sub>4</sub> .	Crystalline form.	Solubilities.	Rosenmund-Kuhnemann iodine No.	
					Theoretical.	Found.
Cholesterol.	Melts clear at 148°.	-38.4	Leaflets from alcohol; dendrites from acetone.	Hot alcohol, warm acetone, cold chloroform, or tetrachloride.	65.7	68.6
Dicholesteryl ether.	Melts turbid at 203°; clear at 209°. Re-melts turbid at 196°; clear at 209°.	-40.8	Trichitic needles from tetrachloride-alcohol.	Cold chloroform or tetrachloride. Insoluble alcohol or acetone.	67.3	69.1
Cholesteryl <i>d</i> -bornyl ether.	Melts clear at 177°.	-17.0	Leaflets or trichitic needles from ether-alcohol.	" "	48.6	51.6
Cholesteryl phenyl ether.	" " 155°.	-28.6	Rectangular leaflets or trichitic needles from ether-alcohol.	" "	54.9	66.4
Cholesteryl allyl ether.	" " 78°. Color play on cooling. Melting point changes with age.	-32.7	Acicular leaflets from acetone.	Hot alcohol or acetone; cold chloroform or tetrachloride.	119.0	111.7
Cholesteryl secondary octyl ether.	Melts clear at 89°.	-24.8	Acicular leaflets from alcohol.	" "	50.9	51.7

Cholesteryl <i>n</i> -heptyl ether.	Melts clear at 96°. Very faint color play on cooling.	-25.6	Acicular leaflets and dendritic needles from alcohol.	Hot alcohol or acetone; cold chloroform or tetrachloride.	52.4	53.4
Cholesteryl <i>n</i> -hexyl ether.	Melts turbid at 68°; clear at 79°. Color play on cooling.	-26.0	Acicular leaflets from acetone.	"	54.0	54.6
Cholesteryl isoamyl ether.	Melts turbid at 94°; clear at 98°.	-28.4	Needles from alcohol.	"	55.6	56.2
Cholesteryl isobutyl ether.	Melts clear at 113°.	-28.7	"	"	57.4	58.2
Cholesteryl <i>n</i> -butyl ether.	Melts turbid at 79°; clear at 86°. Color play on cooling.	-28.8	" acetone.	"	57.4	59.0
Cholesteryl iso-propyl ether.	Melts clear at 132°.	-31.1	Trichitic needles, acicular leaflets, or dendritic needles from ether-alcohol.	Cold chloroform or tetrachloride. Insoluble alcohol or acetone.	59.3	61.8
Cholesteryl <i>n</i> -propyl ether.	" " 100°. Color play on cooling.	-31.0	Variform leaflets or needles from alcohol.	Hot alcohol or acetone; cold chloroform or tetrachloride.	59.3	61.5
Cholesteryl ethyl ether.	Melts clear at 89°. Intense color play on cooling.	-33.0	Long needles from acetone.	"	61.3	62.5
Cholesteryl methyl ether.	Melts clear at 82°. Very faint color play on cooling.	-40.5	Large acicular leaflets from acetone.	"	63.4	65.4

place only in certain solvents, such as carbon tetrachloride and the benzenes; not in the lower alcohols including butyl. However, it is evident from the details given in connection with the above mixed ether syntheses that with increasing molecular weight the alcohols exert less and less retarding action on catalysis occurring in hydrocarbon solutions. Indeed, beginning with isoamyl alcohol, a slight action can take place even when no hydrocarbon is present. Octyl alcohol lends itself, even readily, to the production of cholesteryl octyl ether without the use of any accelerating solvent.

#### SUMMARY.

1. The action of floridin on cholesterol is a dehydrating effect, not a polymerizing effect, as previously suggested. The resulting product, dicholesteryl ether, is formed not only by floridin, but by many other fuller's earths of widely different catalytic activity; also by kieselguhr, kaolin, bentonite, and bauxite, and to a slight extent by talc. No catalytic action was exhibited by pumice, silica gel, natural amorphous silica, permutit, powdered glass, bone-black, or vegetable charcoal.

2. The catalysis does not occur in solutions of the lower alcohols; but in the higher alcohols, from isoamyl to secondary octyl, cholesteryl mixed ethers are formed. However, the mixed ethers are best prepared by conducting the catalysis in benzene, toluene, or xylene, to which has been added a suitable amount of some alcohol or phenol. In this manner the following mixed ethers were synthesized: cholesteryl *d*-bornyl, phenyl, allyl, secondary octyl, *n*-heptyl, *n*-hexyl, isoamyl, isobutyl, *n*-butyl, isopropyl, *n*-propyl, ethyl, and methyl ethers. The tertiary amyl compound could not be formed, on account of decomposition of the alcohol.

3. When an ester of cholesterol, namely the acetate, is treated with floridin, the ester structure is ruptured, and dicholesteryl ether is formed. From the latter the mixed ethers may be prepared, the same as from cholesterol.

4. The mixed ethers of cholesterol are colorless, crystalline bodies, for which the melting point phenomena, optical rotations, crystalline forms, solubilities, Rosenmund-Kuhnnehn iodine numbers, and special properties have been described.

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## ANTIRICKETIC SUBSTANCES.

### V. THE ACTION OF ULTRA-VIOLET RAYS ON THE ETHERS AND ESTERS OF CHOLESTEROL.

By CHARLES E. BILLS AND FRANCIS G. McDONALD.

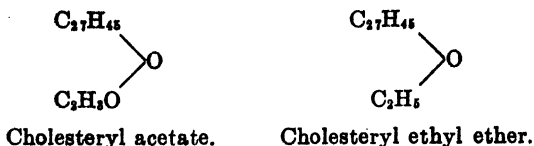
(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.)

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Although the constitution of the cholesterol molecule is as yet but imperfectly understood, chemists are familiar with its double bond and secondary alcohol group. The rôle of these two structures in the activation of cholesterol by ultra-violet rays has been investigated by Hess, Weinstock, and Sherman (1), and Rosenheim and Webster (2). It is agreed that the double bond must be intact if activation is to occur, since all of the saturated sterol compounds investigated do not respond to irradiation. However, the situation in regard to the alcohol group is not so plain.

Hess, Weinstock, and Sherman found that when the alcohol group was altered by acetylation, the resulting cholesteryl acetate became antiricketic upon irradiation, just as did cholesterol itself. Rosenheim and Webster observed that cholesteryl chloride, unlike the organic esters, could not be activated. Similarly, the ketone, cholestenone, and the unsaturated hydrocarbon, cholestene, remained inactive. But Rosenheim and Webster did find that cholesteryl acetate and cholesteryl palmitate exhibited antiricketic activity after irradiation. For some reason not clear to us it was suggested that these esters might have been hydrolyzed by the rays; that free cholesterol, not the ester *per se*, was the substance activated.

The chemical similarity of cholesterol esters and ethers is recalled by the following formulas. It is to be noted that biologically a series of such compounds reveals significant differences.



Elsewhere in this *Journal*<sup>1</sup> we described the synthesis of the fourteen cholesteryl ethers studied in this experiment. The four esters were obtained as follows.

Cholesteryl acetate was prepared by boiling cholesterol with a large excess of acetic anhydride, and recrystallizing the product from pure acetone until the melting point was constant at 115°. Acetone was used in preference to alcohol as a solvent in order to avoid even the remote possibility of hydrolyzing the ester by alcoholysis. (Certain esters, such as methyl tartrate, are thus decomposed (3).) Actually, this precaution was probably unnecessary, for a portion of the purified acetate after boiling with 95 per cent alcohol for 1 hour gave no precipitate with digitonin. By analogous means cholesteryl isobutyrate was obtained in remarkably perfect crystals melting at 128°. Cholesteryl benzoate was prepared by the action of benzoyl chloride on a pyridine solution of cholesterol (4). After recrystallization from ethyl acetate it melted typically at 145° to a turbid liquid clearing at 179° and exhibiting color play on cooling. Cholesteryl cinnamate was obtained by recrystallizing the Kahlbaum product from ligroin until the leaflets were perfectly white and melted at 161–162° with the characteristic play of colors.

Biological tests for antiricketic action were performed as usual by administering the test substance in McCollum's Diet 3143. Experience with over 3000 rats convinces us that Diet 3143 leaves little to be desired as a means of producing uniformly severe and extensive decalcification. In our opinion, failure to obtain satisfactory rickets with this diet generally indicates faulty environmental influences in the rattery or abnormal conditions in the breeding stock. The physical texture of Diet 3143 is much improved, and its biological action in no way impaired, by substituting for the soft wheat recommended by McCollum the hard, whole wheat meal manufactured by Pillsbury (Pillsbury's "100 per cent" flour). Normal young rats 24 days of age develop severe rickets in 18 days on this diet, when kept in a darkened room at 25° ± 3°.

The esters and ethers of cholesterol were tested for antiricketic

<sup>1</sup> Bills, C. E., and McDonald, F. G., *J. Biol. Chem.*, 1927, lxxii, 1.

activity before and after irradiation, at a level of  $\frac{1}{2}$  per cent. The rickety rats were fed the modified diet for a test period of 5 days. Their tibias were examined by the usual line test procedure. To incorporate the sterols in the diet, they were dissolved in ether containing a little seal oil (sufficient to equal 1 per cent of the modified diet), and this solution was evaporated onto the food. The seal oil was used to prevent the sterols crystallizing out on the dry mixture, and to protect the antiricketic products from too rapid deterioration.

All the four esters and fourteen ethers were without calcifying action prior to irradiation. As a source of ultra-violet rays we employed a 220 volt Uviarc operating at 170 volts. This light source is said to be about 2.5 times as powerful as the 110 volt lamps commonly used for therapeutic purposes. In every experiment the burner was 36 cm. above the Petri dish containing the sterol.

Cholesteryl acetate and cholesteryl isobutyrate in the form of fine powder evenly distributed in shallow layers were irradiated for 15 minutes. They became intensely antiricketic. An aromatic ester, the benzoate, was similarly exposed, and this, like the two aliphatic esters, induced heavy calcification at the dose indicated. No attempt was made to determine minimum dosages; for the present it seemed sufficient to administer generous amounts of the various substances, so as to obtain the most definite qualitative distinctions.

Rosenheim and Webster found that the trebly unsaturated sterol, ergosterol, is readily activated. It seemed of interest, therefore, to investigate a cholesterol ester containing an additional double bond in the *acid radical*. Such a substance is cholesteryl cinnamate. This ester, irradiated as above for 15 minutes, induced no healing. A second portion of the cinnamate was exposed for 1 hour, and fed to two rats. From Table I it will be seen that Rat 2729 showed no healing whatever, and Rat 2728 only the merest trace, perhaps accidental. It is thus evident that the cinnamate radical inhibits the activation of cholesterol by ultra-violet rays. The peculiar behavior of cholesteryl cinnamate is excellent evidence for believing that in the case of those esters which do become activated, it is the irradiated ester itself, and not cholesterol liberated therefrom by radiant energy, that constitutes



TABLE I.

*A Partial Record of Tests with Cholesterol Esters and Ethers, and with Cinnamic Acid.*

Unless otherwise specified, all irradiated materials were exposed, in the dry state, for 15 minutes.

Rat No.	Preparation administered.	Grade of test.	Weight.	
			gm.	gm.
2527	Cholesteryl acetate.	—	72-74	6.6
2547	“ “ irradiated.	++++	53-58	6.2
2612	“ “ “	++++	71-81	10.0
2542	Cholesteryl isobutyrate.	—	70-70	7.2
2544	“ “ irradiated.	++++	75-77	9.0
2668	“ “ “	++++	80-80	7.8
2613	Cholesteryl benzoate.	—	75-80	8.2
2614	“ “ irradiated.	++++	71-75	8.4
2615	“ “ “	++++	76-80	8.4
2543	Cholesteryl cinnamate.	—	77-81	8.4
2545	“ “ irradiated.	—	71-79	9.0
2676	“ “ “	—	82-84	8.4
2677	“ “ “	—	74-77	7.4
2728	“ “ “ 1 hr.	—(?)	63-70	7.2
2729	“ “ “ 1 “	—	65-73	7.6
2666	Cinnamic acid.	—	62-70	7.4
2664	“ “ irradiated.	—	56-60	6.2
2665	“ “ “	—	56-61	6.2
2662	“ “ “ in solution.	—	52-56	6.0
2663	“ “ “ “ “	—	54-58	6.0
2541	Cholesteryl methyl ether.	—	68-71	6.2
2556	“ “ “ irradiated.	—	51-53	6.0
2525	Cholesteryl ethyl ether.	—	67-68	5.4
2549	“ “ “ irradiated.	—	69-76	7.6
2539	Cholesteryl isopropyl ether.	—	67-70	7.0
2551	“ “ “ irradiated.	—	56-65	8.4

TABLE I.—*Concluded.*

Res. No.	Preparation administered.	Grade of test.	Weight.		Average daily consumption.
			gm.	gm.	
2530	Cholesteryl <i>n</i> -propyl ether.	—	70-75	6.6	
2552	“ “ “ irradiated.	—	71-83	9.2	
2531	Cholesteryl isobutyl ether.	—	68-72	6.0	
2334	“ “ “ irradiated.	—	62-63	5.6	
2534	Cholesteryl <i>n</i> -butyl ether.	—	55-62	5.8	
2553	“ “ “ irradiated.	—	66-69	7.0	
2528	Cholesteryl isoamyl ether.	—	72-75	6.8	
2201	“ “ “ irradiated.	—	65-70	7.0	
2202	“ “ “ “	—	71-80	7.4	
2616	“ “ “ “ 1 hr.	—	66-66	7.2	
2617	“ “ “ “ 1 “	—	73-75	8.8	
2678	“ “ “ “ in solution.	—	70-75	7.8	
2679	“ “ “ “ “ “	—	66-68	6.6	
2535	Cholesteryl <i>n</i> -hexyl ether.	—	55-62	6.8	
2318	“ “ “ irradiated.	—	54-55	5.4	
2533	Cholesteryl <i>n</i> -heptyl ether.	—	59-62	6.4	
2315	“ “ “ irradiated.	—	66-67	4.8	
2532	Cholesteryl secondary octyl ether.	—	60-63	5.8	
2554	“ “ “ “ irradiated.	—	66-74	8.6	
2526	Cholesteryl phenyl ether.	—	77-77	6.0	
2555	“ “ “ irradiated.	—	73-82	8.6	
2529	Cholesteryl <i>d</i> -bornyl ether.	—	76-80	7.4	
2548	“ “ “ irradiated.	—	64-68	6.8	
1733	Dicholesteryl ether.	—	71-71	6.6	
1750	“ “ irradiated.	—	95-96	8.8	
2540	Cholesteryl allyl ether.	—	70-72	6.8	
2550	“ “ “ irradiated.	—	61-70	8.4	

the antiricketic substance. It would be far fetched to assume that cholesteryl cinnamate liberates cholesterol much less readily than the other esters.

The most plausible explanation of the behavior of cholesteryl cinnamate is that the double bond of the cinnamate radical appropriates the ultra-violet energy required for the alteration of the cholesteryl double bond. Indeed, it is well established (5, 6) that cinnamic acid is altered by irradiation. Under certain conditions various kinds of isomerization take place; under others, polymerization. It would appear that cinnamic acid is altered by rays of the same wave-length as those which activate cholesterol. Does it follow that cinnamic acid is thereby activated?

Cinnamic acid (Eastman) was fed at  $\frac{1}{2}$  per cent. It induced no healing. Portions of the acid were then irradiated dry, and in alcoholic solution, for 15 minutes. They remained without action. We are under the impression that all the rats which received cinnamic acid exhibited rickets of augmented severity. It is evident that when an unsaturated compound absorbs, and is altered by, the antiricketic rays, the compound does not necessarily become antiricketic.

The fourteen cholesterol ethers comprised cholesteryl methyl, ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, isoamyl, *n*-hexyl, *n*-heptyl, and secondary octyl ethers, which may be regarded as the aliphatic types; cholesteryl phenyl ether, cholesteryl *d*-bornyl ether, and dicholesteryl ether, as aromatic types; and cholesteryl allyl ether, as a doubly unsaturated type.

Each of these ethers was irradiated 15 minutes in the dry state. In addition, one ether (the isoamyl) was irradiated dry for 1 hour and, dissolved in a mixture of absolute alcohol and ether, for 15 minutes. No antiricketic activity was developed in any case. Even cholesteryl allyl ether, distinguished by its instability in the dry state, did not become antiricketic.

It is barely possible that activated sterols exert their antiricketic influence only when their alcohol group is free. In the case of those esters which become active upon irradiation, it is conceivable that the activated ester is hydrolyzed in the animal body by the cholesterol esterases which are known to exist, so that active cholesterol becomes the agent of calcification. Perhaps the ethers also are activated, but not made available by the enzymes.

Lack of facilities for spectrographic study of the ethers has prevented us from elucidating this point. A more probable interpretation of our experiments is simply that the ethers of cholesterol, unlike the esters, are not altered by ultra-violet rays.

#### SUMMARY.

1. Among the esters of cholesterol the acetate, isobutyrate, and benzoate are readily converted into antiricketic modifications by the action of ultra-violet rays. Cholesteryl cinnamate is not appreciably activated, but there is evidence that the molecule is nevertheless altered, the alteration taking place at the double bond of the cinnamate radical, instead of in the cholesteryl radical.

2. The antiricketic rays probably induce molecular changes in ordinary cinnamic acid, but they do not render cinnamic acid antiricketic.

3. In the activation of cholesterol esters, the ester molecule as such, and not cholesterol liberated therefrom, is the substance activated.

4. Fourteen cholesterol ethers of widely divergent structure failed to become antiricketic upon irradiation. Whether they underwent molecular alteration of any kind has not been determined.

5. In the activation of sterols both the double bond and the alcohol group are involved. The former must be intact; the latter may be replaced by certain acid radicals.

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## CRYSTALLINE GUANINE NUCLEOTIDE.

BY MARY V. BUELL AND MARIE E. PERKINS.

(From the Laboratory of Physiological Chemistry, the Johns Hopkins University, Baltimore.)

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### INTRODUCTION.

In 1894 Hammarsten (1) prepared from the pancreas a substance which he called  $\beta$ -nucleoprotein to distinguish it from  $\alpha$ -nucleoprotein, a more complex substance. Since that time a great deal of experimental work has been done on the non-protein portion of  $\beta$ -nucleoprotein, to which the name guanylic acid has been given. It is now agreed that guanylic acid is a dehydrolysis product of guanine, *d*-ribose, and phosphoric acid. The analysis by Levene and Jacobs (2) of the crystalline brucine salt of guanylic acid indicated that the formula for the free acid was  $C_{11}H_{14}N_5PO_8$ . These considerations placed guanylic acid, from a chemical view-point, among the nucleotides, and the compound is logically called guanine nucleotide.

The first successful isolation of any nucleotide from yeast nucleic acid was made by Jones (3) who prepared guanine nucleotide and showed that the compound behaved identically with a specimen of guanylic acid prepared from the  $\beta$ -nucleoprotein of the pancreas. Although the guanine nucleotide preparations of Jones and his coworkers (4-6) were amorphous, analysis led to the appropriate theoretical formula for guanine nucleotide and these amorphous preparations yielded crystalline brucine salts which gave correct analyses. However satisfactory may be the analysis of an amorphous compound, prepared under carefully controlled conditions, the conversion of that compound into the crystalline condition before analysis is highly desirable.

It seemed to be a definite contribution therefore when Levene (7) reported the preparation of guanylic acid in crystalline form. No distinctly new procedure was introduced into the method previously used. "It crystallized in the form of long prismatic needles having the same appearance as guanosine. The substance had all the properties of guanylic acid. It gelatinized in the presence of mineral impurities. A test for the presence of free phosphoric acid was negative. It had no melting point, but turned brown at 208°C. The optical rotation in water solution was  $[\alpha]_D^{20} = -7.5$ , and in a 5 per cent ammoniacal solution  $[\alpha]_D^{20} = -43.5$ . On hydrolysis it gave guanosine or guanine sulfate depending on the conditions of the experiment. The free acid, when air-dry, crystallized with 2 mols of

crystal water, and, when dried under diminished pressure at the temperature of a toluene vapor bath to constant weight, still retained  $\frac{1}{2}$  mol of crystal water." The evidence adduced for this latter statement is the fair agreement between the found values for C, H, N, and P and those calculated on the assumption that the compound analyzed actually retained  $\frac{1}{2}$  molecule of water of crystallization.

Apparently there was some difficulty in the method of preparation of crystalline guanylic acid described by Levene, for he reported in a later paper (8), "Generally an amorphous, somewhat gelatinous precipitate settles out. In some instances the solution turns into a semiliquid jelly. To bring about final crystallization, no general rule can be given. At times repeated precipitation with lead acetate will lead to a filtrate which, on concentration, solidifies into a crystalline mass. Often it is advisable to precipitate the nucleotide by means of lead acetate fractionally. The later fractions as a rule crystallize with less difficulty." Again, when manipulating the products of hydrolysis of yeast nucleic acid, Levene (9) analyzed the crystalline brucine salt of guanylic acid instead of the free acid.

Attempts to prepare crystalline guanine nucleotide have met with uncertain success in this laboratory. Occasional crystalline preparations (Jones and Perkins, unpublished data) have not yielded satisfactory analyses. When heated in a drying oven the water of crystallization was not given off sharply. The preparation gradually turned brown and a solution of this product gave reactions for free purines. Without definite knowledge of the water of crystallization no theoretical values could be calculated for the percentage of N, P, and C.

In the course of some of our work the preparation of crystalline guanine nucleotide became a matter of importance. A method of preparation was finally evolved which proved to be successful, simple, and consistent. The pure product obtained was analyzed and its properties were found to be sufficiently different from those described by Levene to warrant the present report.

#### EXPERIMENTAL.

##### *Method.*

50 gm. of Merck's yeast nucleic acid were hydrolyzed at room temperature overnight with 1 per cent sodium hydroxide according to the method of Steudel and Peiser (10) as used by Jones and Perkins (11). The mixed nucleotides were precipitated as the lead salts, the lead was removed with hydrogen sulfide, and the

resulting filtrate was evaporated under diminished pressure at 40–45°C. (outside bath) to the consistency of a syrup. Concentrated ammonium hydroxide was added to neutrality toward litmus, followed by 2 volumes of absolute alcohol. The ammonium salt thus precipitated was collected after several hours and dissolved in a minimum quantity of boiling water. Certain inorganic impurities, insoluble even in large volumes of water, were disregarded. 2 volumes of alcohol were added again, and this process of solution and reprecipitation of the ammonium salt was repeated twice (making a total of four precipitations) with the difference that in the final precipitation only 1 volume of alcohol was used. The product obtained was dissolved in hot water, the solution was filtered, acidified with acetic acid, and treated with a solution of neutral lead acetate as long as a precipitate formed. The lead salt was washed once with hot water, the washings being allowed to cool before filtration, and was then decomposed with hydrogen sulfide and the filtrate aerated. The lead salt was reprecipitated, washed repeatedly until it was shown to be free from ammonia, and decomposed with hydrogen sulfide. The aerated filtrate was treated with silver nitrate, at room temperature, as long as a precipitate formed. This silver precipitate was washed with warm water until further washing was prevented by the colloidal nature of the compound. It was then suspended in warm water, decomposed with hydrogen sulfide, and the aerated filtrate was evaporated under reduced pressure at a temperature of 40°C. (outside water bath) to a small volume, from which crystallization was allowed to take place. The snow-white, beautifully crystalline compound was recrystallized from hot water.

#### *Comments.*

If the preparation was carried out properly and the final solution was sufficiently concentrated, crystallization took place quickly. In fact, it some times began in the vacuum flask. If the separation of the guanine nucleotide from other compounds was not sharp, crystallization was delayed or prevented, or a different type of crystal was obtained. Pure specimens always crystallized as the long tapering needles illustrated in the photomicrograph (Fig. 1). Impure specimens crystallized wholly or in part as short prismatic



needles. Sometimes these short needles were converted into the long tapering needles on long standing with the mother liquor. They could be converted into the desired crystal form if they were dissolved and carried through the silver precipitation once more.

*It is evident that the success of this method lies in the separation effected by the final precipitation of the compound as the silver salt. What the impurities so separated may be is problematical, but there is experimental evidence that one of them is adenine nucleo-*



FIG. 1. Guanine nucleotide  $\times 500$ .

tide. Our purest preparations of adenine nucleotide, when dissolved in water, gave no precipitate when treated with silver nitrate, whereas guanine nucleotide was readily precipitated. This point was investigated in the course of one preparation of guanine nucleotide as follows: The filtrate from the silver procedure was freed from excess silver with hydrogen sulfide, and in the filtrate, after hydrolysis with sulfuric acid, adenine was found and identified. Further evidence is furnished by the following experiment. A saturated solution of crystalline guanine nucleo-

tide was divided into two parts. To one part a small amount of crystalline adenine nucleotide was added. The result was a slow and incomplete crystallization, and the crystals which did form were the short prismatic type. This preparation was carried through the silver procedure with the result that crystallization took place satisfactorily.

### *Analysis.*

Pure crystalline guanine nucleotide was found to have 2 molecules of water of crystallization which were given off sharply when the substance was heated at atmospheric pressure to  $118^{\circ}$ . The percentage of water thus found was 8.92 as compared with a theoretical value of 9.02. Further heating at this temperature for an hour caused no more loss of weight. The exact amount of water lost on heating was again taken up on exposure to the air. On account of the peculiar property of Levene's compound of losing exactly  $1\frac{1}{2}$  molecules of water, when heated under diminished pressure at the temperature of a toluene bath, our compound was heated at  $110^{\circ}$  and was found to lose 8.18 per cent of its weight, an amount corresponding to 1.8 molecules of water.

Total nitrogen was determined by the macro-Kjeldahl method; phosphorus by the technique of Jones and Perkins (12).

The free guanine, which was precipitated by ammonia after hydrolysis of the compound for 1 hour with 20 parts of 7 per cent sulfuric acid in a boiling water bath, was weighed.

"Partial phosphorus" is that phosphorus which is split from the compound by hydrolysis for  $2\frac{1}{2}$  hours with 7 per cent sulfuric acid in a boiling water bath.

Specific rotation was determined with the use of a 1 per cent solution and a tube 2 dm. in length, at  $20^{\circ}\text{C}$ . The value found for an aqueous solution was  $-13.5$  as compared with Levene's finding of  $-7.5$ .

	Theoretical for $\text{C}_{10}\text{H}_{14}\text{N}_5\text{P}_2\cdot 2\text{H}_2\text{O}$	Found.
$\text{H}_2\text{O}$ .....	9.02	8.92
N .....	17.54	17.50
Total P .....	7.77	7.74
Partial P .....	7.77	7.36
Guanine .....	37.84	37.37
$[\alpha]_D^{20}$ in water .....		$-13.5$
$[\alpha]_D^{20}$ in 5 per cent ammonia .....		$-48.5$

## SUMMARY.

A method has been described for the easy and certain preparation of crystalline guanine nucleotide from yeast nucleic acid. Analyses of this compound indicate clearly its chemical identity and purity.

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# THE ACTION OF SODIUM CARBONATE ON YEAST NUCLEIC ACID.

By H. O. CALVERY.

*(From the Department of Physiological Chemistry, the Johns Hopkins University, Baltimore.)*

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## INTRODUCTION.

Interest has been renewed of late by the work of Steudel and Peiser (1) and more recently by Jones and Perkins (2) in the number of nucleotides which may be considered of primary origin in the decomposition of yeast nucleic acid by the various hydrolytic agents. The one which has been called in question is uracil nucleotide. It is the resulting product when cytosine nucleotide is deaminized so that the amino group is replaced by hydroxyl. Kowalevsky (3) was the first to suggest that there were only three nucleotides. Levene and Jacobs (4) proposed the tetranucleotide theory when they found all four nucleosides by neutral hydrolysis of yeast nucleic acid, and this was later strengthened by the isolation of crystalline uracil nucleotide itself by Levene (5). But as Jones and Perkins (2) have pointed out, the fact that a certain substance is not changed into another by a given procedure is no criterion that the change will not occur when the original substance is united by chemical union to some other substance. In the latter case the chemical properties may be entirely altered. And the fact that cytosine nucleotide is not converted into uracil nucleotide under the same conditions in which yeast nucleic acid gives uracil nucleotide (6) does not prove that uracil nucleotide is a primary constituent of nucleic acid.

Steudel and Peiser in their article have described a method whereby they meant to settle the question of the origin of the uracil nucleotide. By the process described by them they separated the barium salt of what they supposed was yeast nucleic

acid into several fractions. They used the P:N ratio as a criterion of the composition of each fraction prepared by them. It is obvious that this ratio means very little or nothing when dealing with such a large molecule as yeast nucleic acid. One of these fractions, Fraction A, they considered to be the barium salt of the true nucleic acid and it corresponded to the formula  $C_{29}H_{42}O_{25}N_{13}P_3$ . They considered Fraction E to be the barium salt of uracil nucleotide described by Levene *since it had a similar P:N ratio*.

The object of the present investigation has been to make a more complete study of each of the fractions obtained by Steudel and Peiser and to see if these were not simple mixtures of nucleotides and if their investigation might not throw more light on the structure of the nucleic acid molecule. Also if uracil nucleotide could be removed by this method, it seemed a simple process to separate it from the other nucleotides. Steudel and Peiser gave no definite information concerning the components of the fractions prepared by them. They used material from Boehringer and Söhne and in this investigation the yeast nucleic acid of Merck, who is the American agent for Boehringer and Söhne, has been used.

#### EXPERIMENTAL.

*Preparation of the Fractions of Barium Salts.*—50 gm. of yeast nucleic acid were suspended in a liter of water and just enough dilute sodium hydroxide was added to take the nucleic acid into solution. It still reacted acid to litmus. Lead acetate solution was then added until further addition did not give a precipitate. The precipitate formed by this procedure was filtered off with suction and washed thoroughly. The precipitate was then made into a fine emulsion with 1500 cc. of water and enough 20 per cent sodium carbonate solution was added to precipitate all the lead as lead carbonate. The solution was alkaline, which showed that there was an excess over the amount required to form the sodium salt of nucleic acid. The lead carbonate was filtered off and the clear filtrate was evaporated in a vacuum at a low temperature (50–60°) to a volume of 500 cc. The solution was then made faintly acid with acetic acid and the carbon dioxide driven out by heat. A saturated solution of barium acetate was added and the precipitate which formed was filtered off and suspended in hot

water. The greater part was soluble. The hot solution was filtered and allowed to cool. A precipitate separated which was filtered off, dried with alcohol and then in a desiccator. This was Fraction A and weighed 6.3 gm.

The filtrate was treated with barium acetate solution and on standing overnight a further precipitate separated and was filtered off. This precipitate was suspended in boiling water and filtered from the insoluble part. The clear cool filtrate was treated with alcohol as long as a precipitate formed, filtered, and the precipitate dried in a desiccator. This was Fraction B and weighed 4.8 gm.

The filtrate obtained from the precipitate formed by the second precipitation with barium acetate was treated with alcohol as long as a precipitate formed. The precipitate was filtered off

TABLE I.

Fraction.	Amount. Steudel and Peiser.	Amount. Calvery.	Nitrogen values. Steudel and Peiser.		Nitrogen values. Calvery.	
			I	II	I	II
	gm.	gm.	per cent	per cent	per cent	per cent
A	5	6.3	13.82	13.79	13.52	13.54
B	5	4.8	10.43	10.35	10.19	10.17
C	5	4.5	8.17	8.17	8.19	8.24
D	10	7.0	9.17	9.29	9.25	9.38
E	2.5	1.5	3.77	3.79	3.75	3.55
F		6			13.39	13.38

and suspended in hot water in which it was mostly soluble. It was filtered from any insoluble residue. The barium salts were again thrown out of solution by means of alcohol and dried in a desiccator. This was Fraction C and weighed 4.5 gm.

The insoluble residues from all of the above procedures were combined and treated with dilute sodium hydroxide in which they were mostly soluble. The insoluble part was centrifuged away and dried in a desiccator. This was Fraction E and weighed 1.5 gm. The filtrate was made faintly acid with acetic acid and on standing overnight a large precipitate formed. It was filtered off and dried in a desiccator. It weighed 6 gm. and has been called Fraction F, the part which Steudel and Peiser did not investigate. It was, no doubt, for the most part unhydrolyzed yeast nucleic acid but had carried down some of the free nucleotides with it.

TABLE II.

Source of nucleotide analyzed.		Amount used.	Acid.*	N found.
		gm.	cc.	per cent
Adenine nucleotide of Fraction A. I.				
"	"	0.2176	10.7	19.12
"	"	0.2283	11.3	19.17
"	"	0.1953	9.6	19.20
"	"	0.3176	15.7	19.15
"	"	0.1819	9.0	19.19
"	"	0.2015	9.9	19.11
"	"	0.2652	13.0	19.13
"	"	0.2431	13.0	19.15
"	"	0.1951	9.7	19.24
"	"	0.1842	9.1	19.17
"	"	0.2334	11.5	19.20
"	"	0.2489	12.3	19.21
"	"	0.1735	8.5	19.11
"	"	0.1993	9.9	19.23
"	"	0.1830	9.1	19.25
"	"	0.2183	10.6	19.27
"	"	0.3413	17.1	19.17
"	"	0.3053	18.0	19.13
"	"	0.1713	8.4	19.21
"	"	0.1841	9.1	19.19
"	"	0.1924	9.6	19.30
"	"	0.2372	11.8	19.31
"	"	0.2210	11.0	19.27
"	"	0.2195	10.8	19.11
Guanine nucleotide of Fraction A. I.				
"	"	0.2176	10.7	19.12
"	"	0.2283	11.3	19.17
"	"	0.1953	9.6	19.20
"	"	0.3176	15.7	19.15
"	"	0.1819	9.0	19.19
"	"	0.2015	9.9	19.11
"	"	0.2652	13.0	19.13
"	"	0.2431	13.0	19.15
"	"	0.1951	9.7	19.24
"	"	0.1842	9.1	19.17
"	"	0.2334	11.5	19.20
"	"	0.2489	12.3	19.21
"	"	0.1735	8.5	19.11
"	"	0.1993	9.9	19.23
"	"	0.1830	9.1	19.25
"	"	0.2183	10.6	19.27
"	"	0.3413	17.1	19.17
"	"	0.3053	18.0	19.13
"	"	0.1713	8.4	19.21
"	"	0.1841	9.1	19.19
"	"	0.1924	9.6	19.30
"	"	0.2372	11.8	19.31
"	"	0.2210	11.0	19.27
"	"	0.2195	10.8	19.11

Guanine nucleotide of Fraction D. I. ....				0.1631	8.1	19.36
"	"	"	II. ....	0.1912	9.5	19.32
"	"	"	E. I. ....	0.1874	9.3	19.22
"	"	"	" II. ....	0.1876	9.25	19.17
"	"	"	F. I. ....	0.2516	12.45	19.31
"	"	"	" II. ....	0.2348	11.6	19.21
"	"	from A after hydrolysis with NaOH. I. ....		0.2676	13.2	19.22
"	"	" " " II. ....		0.2241	11.1	19.18
"	"	" C " " I. ....		0.2222	11.3	19.28
"	"	" " " " II. ....		0.2183	10.8	19.23
"	"	" hydrolysis with Na <sub>2</sub> CO <sub>3</sub> . I. ....		0.3177	15.6	19.13
"	"	" " " II. ....		0.3358	16.6	19.24
Cytosine nucleotide from combined Fraction A-F. I. ....				0.1763	5.9	13.01
"	"	" " " II. ....		0.1852	6.2	13.09
"	"	" A after hydrolysis with NaOH. ....		0.2050	6.8	12.87
"	"	" C " " " ....		0.2035	6.9	13.20
"	"	" hydrolysis with Na <sub>2</sub> CO <sub>3</sub> . I. ....		0.2989	9.9	12.93
"	"	" " " II. ....		0.3692	12.2	12.95

\* 1 cc. of standard acid = 0.003884 gm. of nitrogen.



The filtrate from Fraction F was treated with alcohol and the precipitate which formed was filtered off and dried in a desiccator. It was Fraction D and weighed 5 gm.

The relationship between the per cent of nitrogen obtained by the present investigator and that of Steudel and Peiser is shown in Table I.

*Preparation of Guanine Nucleotide and Adenine Nucleotide from Fraction A.*—25 gm. of the material obtained in Fraction A were suspended in hot water and filtered from a small residue which did not go into solution. Lead acetate was then added to the clear filtrate until no further precipitate formed when more lead acetate was added to a portion of the cooled solution. The lead salt was filtered off and washed thoroughly. It was then suspended in hot water and the lead precipitated as lead sulfide. The lead sulfide was filtered off and the excess hydrogen sulfide removed by aeration. The solution was then concentrated to a small volume in a vacuum at a low temperature. It was further concentrated to a syrup in a vacuum desiccator and hardened with absolute alcohol. The hardened material was dissolved in twice its weight of water, neutralized with ammonia, and treated with 1.5 times its weight of absolute alcohol. This procedure separated the nucleotides into two fractions, one of which is known as the guanine fraction and the other, the adenine fraction. The insoluble guanine fraction was filtered off and the nucleotides of the adenine fraction were taken through the usual lead procedure, and on evaporation of the filtrate from the lead sulfide adenine nucleotide crystallized out. This was recrystallized once from water and analyzed. The analysis is given in Table II. The filtrate from the adenine nucleotide was concentrated in a vacuum desiccator to a small volume and treated with a hot saturated solution of brucine in 95 per cent alcohol. On cooling, a heavy precipitate of brucine salts separated. The brucine salts were recrystallized once from 35 per cent alcohol and the filtrate used to prepare more adenine nucleotide in the usual way, by removal of the brucine with ammonia, the subsequent conversion of the ammonium salt into the lead salt, and the removal of the lead with hydrogen sulfide.

The precipitated ammonium salts of the guanine fraction were dissolved in twice their weight of water and again precipitated by

addition of an equal volume of absolute alcohol. This process was repeated a second time in order to insure complete removal of all other nucleotides. The guanine nucleotide was prepared by conversion of the ammonium salt into the lead salt and removal of the lead by means of hydrogen sulfide. The filtrate from the lead sulfide was concentrated in a vacuum to a small volume and on cooling the guanine nucleotide separated as a white amorphous powder. The analysis is given in Table II.

The procedure for the preparation of guanine nucleotide and adenine nucleotide from Fractions B, C, D, E, and F was identical with that used in Fraction A, and the analyses are given in Table II.

*Hydrolysis with Dilute Sodium Hydroxide.*—The exact procedure was followed as was used by Jones and Perkins (2). Dilute sodium hydroxide completely hydrolyzes yeast nucleic acid at room temperature with the formation of the nucleotides. This mixture of nucleotides was neutralized and carried through the same procedure described above for the preparation of the fractions of barium salts. Only Fractions A and C were obtained and guanine, adenine, and cytosine nucleotides were prepared from each fraction in the usual way.

*Cytosine Nucleotide.*—The brucine salts obtained from the adenine fraction of each of the above fractions of barium salts were combined after the first recrystallization and recrystallized eight times from 35 per cent alcohol. After each recrystallization part of the precipitate was dried and analyzed. After the fifth recrystallization it had the composition of the brucine salt of cytosine nucleotide and did not change on further recrystallization. The results of these analyses are shown in Table III. The final residue was combined with the precipitate obtained by evaporation of each of the filtrates after the fifth recrystallization, suspended in water, and the brucine removed by addition of ammonia, filtering, and extracting the filtrate with chloroform. The free nucleotide was prepared as usual, and on concentration of the solution in a vacuum desiccator beautifully crystalline cytosine nucleotide separated. The analysis is given in Table II.

*Hydrolysis with Sodium Carbonate.*—50 gm. of yeast nucleic acid were suspended in a liter of water and enough saturated sodium carbonate solution was added to neutralize the solution.

A further addition of enough 20 per cent sodium carbonate was made to make a 5 per cent solution. This solution was heated at 50–60° on a water bath for 4 to 5 hours. At the end of this time the usual hydrochloric acid test showed complete hydrolysis. The solution was cooled and carefully made faintly acid with

TABLE III.

Crystallization No.	Recovered from mother liquor.	Sample.	Acid.*	Nitrogen.
	<i>gm.</i>	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>
1	6.2	0.2676	6.4	9.16
2	2.4	0.2134	4.8	8.71
3	1.0	0.3232	6.9	8.06
4	0.85	0.3173	6.6	8.15
5	0.28	0.2886	5.9	7.92
6	0.32	0.2152	4.3	7.81
7	0.34	0.2343	4.7	7.88
8	0.26	0.2181	4.4	7.91

\* 1 cc. of standard acid = 0.003884 gm. of nitrogen.

TABLE IV.

Crystallization No.	Recovered from mother liquor.	Sample.	Acid.*	Nitrogen.
	<i>gm.</i>	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>
1	10.13	0.2162	5.8	9.46
2	4.32	0.2811	6.4	8.82
3	2.5	0.2191	4.7	8.31
4	2.9	0.3244	6.8	8.11
5	0.95	0.3422	7.05	8.02
6	0.62	0.3410	6.9	7.89
7	0.56	0.2571	5.25	7.94
8	0.58	0.2319	4.7	7.83
9	0.47	0.3120	7.3	7.85

\* 1 cc. of standard acid = 0.003884 gm. of nitrogen.

acetic acid and the nucleotides precipitated with lead acetate. They were carried through the ordinary process of separation into two fractions and the preparation of the free nucleotides. The brucine salts of the adenine fraction were recrystallized nine times from 35 per cent alcohol. The results of these analyses after each recrystallization are given in Table IV. After the sixth recrystallization the precipitate had the composition of the brucine

salt of cytosine nucleotide. All three nucleotides were prepared and their analyses are given in Table II.

#### DISCUSSION.

In order to obtain the fractions for investigation the procedure of Steudel and Peiser was followed as closely as possible. The similarity of the fractions of the two investigators may be noted in a comparison of the nitrogen analyses in Table I. The only point at which we seem to differ is in the size of the precipitate, which the present investigator found to be one of the largest and which has been designated as Fraction F, but which Steudel and Peiser found to be extremely small and was discarded by them. They did not believe that the reagents or conditions which were used by them could in any way alter the nucleic acid molecule. But in view of the more recent work of Steudel and his associates (7) and of Jones and Perkins (2) it seems reasonable to expect that, if cold dilute sodium hydroxide would hydrolyze yeast nucleic acid into its nucleotides, the warm sodium carbonate solution which was evaporated by the German workers would produce the same effect. The author believed that this was what had occurred and that each fraction would prove to be a mixture of nucleotides. A preliminary investigation showed that each fraction did contain guanine and adenine, some fractions more than others. The subsequent isolation and analysis of guanine nucleotide and adenine nucleotide from each fraction proved conclusively that hydrolysis had occurred in the procedure. No attempt was made to prepare cytosine nucleotide and uracil nucleotide from each fraction since they are much more difficult to obtain. However, the brucine salt residues from the adenine nucleotide in each fraction were combined and recrystallized nine times from 35 per cent alcohol. The residue had the composition of the brucine salt of cytosine nucleotide after the fifth crystallization and did not change on further recrystallization. When the last four residues from the mother liquors were combined with the final product and carried through the usual procedure for the preparation of free nucleotides, crystalline cytosine nucleotide was obtained. There was no evidence of uracil nucleotide. Steudel and Peiser did not investigate and failed to observe that Fraction E contained a carbonate which was probably barium carbonate

and no doubt was formed during the long procedure from the carbon dioxide of the air. This, with a small amount of inorganic phosphate could easily be responsible for the low N:P ratio obtained in this fraction. The above is conclusive proof that there has been some hydrolysis but not a complete separation by this procedure.

In order to determine whether a separation of the nucleotides might be made by this process some nucleic acid was completely hydrolyzed with dilute sodium hydroxide at room temperature and taken through the same procedure. Under these conditions only two of the above fractions, namely Fractions A and C, were obtained, and guanine, adenine, and cytosine nucleotides were prepared from each. In the first case there had not been complete hydrolysis.

The foregoing evidence sustains the belief that sodium carbonate will hydrolyze yeast nucleic acid with the production of only three nucleotides. As final proof of this the action of sodium carbonate alone was tried. It was found that 5 per cent sodium carbonate did not produce complete hydrolysis on standing at room temperature for 30 days. But if a solution of yeast nucleic acid in 5 per cent sodium carbonate was heated for 4 to 5 hours on a water bath at a temperature of 50–60°, hydrolysis was complete and the solution gave no precipitate when hydrochloric acid was added. The usual procedure was followed for the preparation of the nucleotides but only guanine, adenine, and cytosine nucleotides were obtained with no evidence of uracil nucleotide. Uracil nucleotide has never been reported in as large yields as the other nucleotides have been and only once has it been reported as the free nucleotide itself (5).

#### SUMMARY.

1. Sodium carbonate at low temperature hydrolyzes yeast nucleic acid into its nucleotides.

2. If uracil nucleotide is formed by this procedure, I have been unable to find it and as Jones and Perkins (2) have pointed out, its properties are such that it would be the nucleotide least likely to be lost.

3. In their attempt to purify yeast nucleic acid Steudel and Peiser did nothing more than juggle the barium salts

of the various nucleotides, and the part they supposed to be the barium salt of uracil nucleotide has been shown definitely to contain adenine nucleotide, guanine nucleotide, and barium carbonate.

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# THE GASOMETRIC DETERMINATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN BLOOD, AND ITS APPLICATION TO BLOOD VOLUME STUDIES.

BY DONALD D. VAN SLYKE AND FRIEDA S. ROBSCHUIT-ROBBINS.

(From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Department of Pathology of The University of Rochester School of Medicine and Dentistry, Rochester, New York.)

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This paper presents a development of the blood carbon monoxide methods of Van Slyke and Salvesen (10) and of Harington and Van Slyke (4) with attention to certain details which reduce the error to 0.02 or 0.03 volumes per cent.

The present procedure was developed primarily to make possible the determination of blood volumes by the carbon monoxide method of Grehant and Quinquaud (3) without saturating, as has previously been necessary (1, 3, 6, 7), as much as one-third of the blood hemoglobin with CO in order to obtain accurate results. A technique requiring the saturation of only one-tenth to one-twentieth of the circulating hemoglobin makes the method more safe and convenient for use with human subjects, and with animals permits the introduction of the CO by intravenous injection of CO-saturated blood instead of by inhalation of the gas.

## I. Determination of Small Amounts of Carbon Monoxide in Blood.

### *Reagents.*

*Acid Ferricyanide Solution.*—This is the same solution used by Van Slyke and Neill for carbon monoxide in blood. It contains 3.0 gm. of Merck's saponin, 8.0 gm. of  $K_3Fe(CN)_6$ , and 40 cc. of 1 N lactic acid made up to 1 liter with water. It will keep for several weeks.

*Pyrogallol.*—10 gm. of pyrogallol are dissolved in a solution of 160 gm. of KOH in 130 cc. of water. For the CO determination



described below, this, because of its almost complete inability to dissolve either  $N_2$  or CO gas, is preferable to hydrosulfite solution as oxygen absorbent. The blood is removed from the chamber before the oxygen is absorbed, so that the solution does not gum the apparatus by precipitating the proteins.

*Glycerol-Salt Solution.*—1 volume of glycerol is mixed with 3 volumes of saturated NaCl solution. This solution, because of its lower viscosity, is more satisfactory than the 1 to 1 mixture used by Harington and Van Slyke. It is freed from air by extraction in the blood gas apparatus (see p. 535 of Van Slyke and Neill), and is stored under paraffin oil in a cylindrical separatory funnel, or, preferably, in the mercury bulb described on the same page. If protected only by oil it must be used on the same half day that it is extracted, as the paraffin oil merely retards the access of air. The solubility of air in this glycerol-salt solution is only a fraction of the solubility in water, but it is sufficient to necessitate the extraction before the solution is used for the present analysis.

*Winkler's cuprous chloride solution* for absorbing CO. 200 gm. of CuCl, 250 gm. of  $NH_4Cl$ , 750 cc. of water. This solution also is freed of air not longer than 4 hours before use, and is kept under a layer of paraffin oil.

*Technique of Analysis.*—In the chamber of the blood gas apparatus described by Harington and Van Slyke (4) are placed 25 cc. of the acid ferricyanide solution and 6 or 7 drops of caprylic alcohol. The air is removed by evacuating, shaking 3 minutes, ejecting the extracted air, and repeating the process once more. 5.5 cc. of the solution are forced up into the cup of the apparatus. Then 5 cc. of blood are run under this layer of solution into the chamber of the apparatus from a pipette provided with a stop-cock and rubber tip, in the manner described by Van Slyke and Neill on p. 532 of their paper (9). 0.5 cc. of the ferricyanide from the cup is then run into the chamber to wash out the cock of the apparatus; the cock is sealed with a drop of mercury, and the 5 cc. of ferricyanide left in the cup are removed.

The mercury in the chamber is now lowered to the 50 cc. mark, and the apparatus is shaken for 8 minutes. Because of the large volume of solution a longer time than the usual 2 or 3 minutes is needed to obtain maximum extraction of the gases. After

extraction is finished the blood-ferricyanide mixture is drawn down into the bulb at the bottom of the chamber in two successive portions and ejected.

The chamber is then washed with two successive portions of about 5 cc. each of air-free glycerol-salt solution, as described by Harington and Van Slyke, to remove the blood-ferricyanide mixture. The oxygen is then absorbed with pyrogallol as follows. The glycerol-salt solution is removed from the chamber, and 5 cc. of pyrogallol are run into the cup of the apparatus. The mercury in the chamber is so adjusted that a gas space of 3 or 4 cc. is present in the latter, and the stop-cock connecting manometer and leveling bulb is closed. The pyrogallol is then admitted into the chamber in a slow trickle at the rate of about 0.5 cc. per minute. The mercury in the manometer falls at first rapidly, then more slowly, as  $\text{CO}_2$  and  $\text{O}_2$  are absorbed. When, at the end of about 2 minutes, the rate of fall has become imperceptible, the cock leading to the leveling bulb is opened, and the rest of the 5 cc. of pyrogallol is admitted under slightly negative pressure. The cock at the top of the chamber is then sealed with mercury, and the final traces of oxygen are absorbed by raising and lowering the leveling bulb so that the pyrogallol rises and falls in the upper fourth of the chamber. After it has been raised and lowered about 5 times the surface of the pyrogallol is brought to the 0.5 mark (or to the 2.0 cc. mark in case there is so much CO that it gives over 400 mm. pressure at 0.5 cc. volume), and the manometer is read. The process and the reading are repeated until the latter becomes constant. Complete absorption of the oxygen requires about 5 minutes in all. The last trace must be removed with certainty, because if any oxygen is left it will be absorbed by cuprous chloride in the next stage of the analysis, and will be calculated as carbon monoxide.

After absorption of the oxygen, the pyrogallol is ejected through the trap at the bottom of the chamber, and the chamber is washed with two successive 5 cc. portions of glycerol-salt solution to remove the alkali, which would otherwise precipitate the cuprous chloride solution added later.

A third portion, this time exactly measured, of 5 cc. of glycerol-salt solution is then run in, and the manometer is read with the gas volume in the chamber at the original 0.5 or 2.0 cc. The

reading is checked by lowering the solution in the chamber a little, and then bringing the meniscus back to the 0.5 cc. mark. Because of the time required for drainage of the glycerol-salt solution down the walls at the top of the chamber above the 0.5 or 2.0 cc. mark, the actual free volume is usually a little under 0.5 or 2.0 cc. at the first reading. The second manometer reading will then be a little lower, but the third or fourth will be constant. The final reading is noted as  $p_1$ . After the final reading the glycerol-salt solution is ejected through the trap at the bottom of the chamber. The  $p_1$  reading over the glycerol-salt solution is used in preference to that over the pyrogallol, because the meniscus of the clear glycerol-salt solution can be located on the 0.5 or 2.0 cc. mark more accurately than the meniscus of the black pyrogallol solution.

To absorb the carbon monoxide, 6 cc. of the air-free Winkler's cuprous chloride solution are placed in the cup of the apparatus, and 5 cc. are run into the chamber at *slight* negative pressure, the mercury surface in the leveling bulb being only a few cm. below that in the chamber, and the cock open between the leveling bulb and the chamber (note remarks by Harington and Van Slyke on p. 582 of their paper). The cuprous chloride is admitted slowly enough so that 2 minutes are taken. In this time absorption of carbon monoxide is complete. The surface of the solution is now lowered carefully to the original 0.5 or 2.0 cc. mark and the pressure  $p_2$  in the manometer is read. The reading may be checked once by lowering the solution in the chamber a few mm. and bringing it back to the mark. If several check readings are attempted, however, a gradual rise in the observed pressures will be noted, because the absorbed CO begins to return to the gas phase, on account of the loose, reversible character of the combination between CuCl and CO. The lowest reading of such a series is the correct one.

The time required for the entire analysis is about 30 minutes.

#### *Calculation of CO Content of Blood.*

The partial pressure of carbon monoxide,  $P_{CO}$ , in mm. of mercury is  $P_{CO} = p_1 - p_2 + 2.0$ .

The 2.0 mm. correction is due chiefly to the fact that the gly-

cerol-salt solution has a vapor tension about 2 mm. lower than the cuprous chloride solution.

The CO content of the blood is calculated by multiplying the observed  $P_{CO}$  pressure by the proper factor from Table I, which is calculated by Van Slyke and Neill's Equation 6 with the fac-

TABLE I.

*Factors by Which  $P_{CO}$  Is Multiplied to Calculate Volume Per Cent of CO When 5 Cc. of Blood Are Analyzed.*

Temperature.	Factor when $P_{CO}$ is measured with gas at 0.5 cc. volume.	Factor when $P_{CO}$ is measured with gas at 2.0 cc. volume.
°C.		
15	0.01277	0.0511
16	73	09
17	67	07
18	62	05
19	57	03
20	52	01
21	48	0.0499
22	43	97
23	38	95
24	34	94
25	29	92
26	24	90
27	19	88
28	15	86
29	11	84
30	06	82
31	02	81
32	0.01198	79
33	93	77
34	88	75

tors in their Table I. In the analysis above described  $a = 0.5$ , or 2.0,  $S = 25.0$ ,  $i = 1.00$ , and cc. sample = 5.0.

In the analysis as above described for each cc. of blood, 4 cc. of the acid ferricyanide solution are used instead of 2.5 cc., as prescribed by Van Slyke and Neill and by Harington and Van Slyke. We have found that in some samples of dog blood the larger amount is necessary.

## II. The Carbon Monoxide Blood Volume Method with Intravenous Injection of the CO.<sup>1</sup>

*Procedure.*—For injection, sufficient blood to make somewhat over 4 cc. per kilo of body weight of the animal to be injected was defibrinated, strained through gauze, and saturated with carbon monoxide gas (made by heating anhydrous formic acid and concentrated sulfuric acid (1, 3)). A cylindrical separatory funnel, of 10 or 20 times the volume of the blood portion, made a convenient saturator. The blood was placed in the funnel, which was then evacuated from the cock as completely as possible by a water pump, and was filled with carbon monoxide at atmospheric pressure. The saturation was accomplished by rotating the funnel for a half hour in the manner shown by Stadie (8). With dogs one may draw the blood sample either from the animal whose blood volume is to be determined, or from another donor.

Part of the saturated blood was reserved for CO determinations on 2 cc. portions by the technique of Van Slyke and Neill. Since the O<sub>2</sub> and N<sub>2</sub> had been displaced from the blood by CO, only the gases CO<sub>2</sub> and CO remained. Consequently, in this analysis of the CO-saturated blood, the gases were extracted, the CO<sub>2</sub> was absorbed with 1 N sodium hydroxide, and the entire residual gas was measured as CO, the *p*<sub>2</sub> reading being taken after ejection of the CO gas. The absence of oxygen and nitrogen made the use of specific absorbents for O<sub>2</sub> and CO unnecessary.

Of the CO-saturated blood a measured volume approximating 4 cc. per kilo of body weight, and equal to about one-twentieth of the animal's blood volume, was injected intravenously in the course of 1 to 1.5 minutes. At intervals of a few minutes thereafter samples of about 20 cc. were drawn from another vein, without exposure to air, as described by Austin *et al.* (2).<sup>2</sup> 5 cc. portions were analyzed for CO content by the special technique described in this paper.

<sup>1</sup> In the procedure described the CO is injected in the form of blood saturated with the gas. Preliminary experiments have shown that intravenous injection of the gas itself is possible, provided the rate is sufficiently slow and uniform to permit absorption of the gas by the blood in the vein.

<sup>2</sup> According to Lee and Whipple, however, this precaution is unnecessary. The affinity of CO for blood is so great that significant loss does not occur even if ordinary contact with air in a tube is permitted.

In order to ascertain also the hemoglobin content of the blood, a portion of 7 cc. of the drawn blood was saturated with CO gas, as described above, and the CO content was determined as in the analysis of the injected blood. To calculate the CO bound by hemoglobin, the physically dissolved CO was estimated and subtracted from the total observed CO content. The physically dissolved CO was estimated on the assumption that the solubility of CO in blood is proportional to the water content of the latter, and is therefore on the average 82 per cent of the solubility in pure water. This assumption is probably not exact, but the degree of error introduced appears to be slight, as hemoglobin contents estimated from CO capacity, with this solubility correction, are the same as those estimated from O<sub>2</sub> capacity after saturation with air. The physically dissolved CO, in volumes per cent of the blood, is thus estimated to be at 15°, 2.16; at 20°, 1.95; at 25°, 1.82; at 30°, 1.71.

The value of the factor,  $f = \frac{\text{cc. cell volume per 100 cc. blood}}{\text{cc. CO capacity per 100 cc. blood}}$ , which is used in calculating the volume of blood cells in the body (see Equation 3 below) was determined as follows. A 10 cc. sample of blood was drawn into a tube containing 2 cc. of 1.6 per cent neutral potassium oxalate (isotonic for blood, as used by Lee and Whipple (5)). 7 cc. of the oxalated blood were used for determination of the CO or O<sub>2</sub> capacity, while the cell volume in other samples was determined by hematocrit. (Instead of blood treated with isotonic oxalate, defibrinated blood may be used to determine  $f$ . In this case the defibrination should be performed without loss of CO<sub>2</sub> in order to prevent the shrinking of several per cent in the cell volume which occurs if by reason of such loss the serum pH rises, as it may, to 8 (Van Slyke, Wu, and McLean, 11)).

#### *Calculation and Interpretation of Blood Volume Results.*

The interpretation of the results of the carbon monoxide method has been made the subject of a thorough experimental and critical study by Whipple and his collaborators (1, 7). No one should apply this blood volume method without consultation of their papers, the conclusions of which will here be but briefly indicated in connection with the calculations.

*CO-Binding Capacity of Total Hemoglobin.*—Assuming that during the interval between injection of CO and withdrawal of the venous blood sample for analysis, (1) distribution of the injected carbon monoxide among the cells of the circulating blood becomes uniform, and (2) none of the injected CO leaves the circulation, either through the lungs or by diffusion into the tissues, one could calculate the CO-binding capacity of the circulating hemoglobin as follows:

$$(1) \text{ Cc. CO-binding capacity of total blood hemoglobin} = (\text{cc. CO injected}) \times \frac{(\text{volume per cent CO capacity of circulating blood})}{(\text{volume per cent CO content of circulating blood})}$$

Distribution of the injected carbon monoxide does, in fact, seem to be completed in 3 or 4 minutes.

Loss from the lungs within this period appears, both from Whipple's results (1) and ours, to be negligible in this time.

Diffusion of CO from blood to tissues, however, is a factor concerning the rapidity and extent of which we have no conclusive data. As Whipple has shown (1, 12) the muscles contain considerable amounts of pigment which when extracted with dilute ammonia and saturated with CO behaves colorimetrically like hemoglobin. Whether in the animal carbon monoxide actually passes from the blood cells to combine with this pigment in the muscles, how rapidly, and in what proportion, are questions that await experimental solution.

In favor of the probability that during a period of 10 minutes but a small proportion of CO passes from blood to tissues, are the observations of Smith, Arnold, and Whipple (7) that the CO method gives *lower* total blood volumes than the dye methods. In their experiments an atmosphere containing CO was respired for 6 minutes, and the blood sample for CO determination was drawn 4 minutes later. The effect of passage of CO to the tissues would be to *increase* the dilution of CO in the blood, and the CO-binding capacity, blood cell volume, and blood volume of the body estimated from the blood CO concentration.

*Volume of Total Blood Cells in Body.*—From the total CO capacity of the body the total volume of blood cells in the body is calculated as

$$(2) \text{ Cc. total blood cells in body} = (\text{cc. total CO-binding capacity}) \times f.$$

The total CO capacity is estimated by Equation 1. The factor  $f$  is the ratio  $\frac{(\text{cc. cell volume per 100 cc. blood})}{(\text{cc. CO capacity per 100 cc. blood})}$ , which can be accurately determined as described above. The uncertainties of the red cell volume estimation are those of total CO capacity factor, discussed in connection with Equation 1.

*Total Blood Volume.*—The formula for estimating total blood volume is

$$(3) \quad \text{Cc. blood volume} = \frac{100 \times (\text{cc. CO injected})}{(\text{volume per cent CO in circulating blood})}$$

This equation is based upon the assumptions of uniform distribution and negligible loss of CO, discussed in connection with Equation 1, and in addition a third assumption; *viz.*, that the volume per cent of cells is constant in all parts of the circulating blood. Smith, Arnold, and Whipple (7) give reasons for believing that this third assumption is not even approximately exact, that the peripheral blood samples drawn for CO analyses contain a larger proportion of hemoglobin than the average blood of the body, and that in consequence the denominator of the fraction

$\frac{\text{CO injected}}{\text{CO content of blood}}$  is larger in the sample than in the average blood of the body. Presumably as a result of this lack of uniformity in the circulating blood, they obtained in dogs by the CO method total blood volumes about 20 per cent lower than they considered correct, when the results were compared with those of dye injection methods. They considered that the most accurate total blood volume was to be obtained by adding the cell volume yielded by the CO method (Equation 2 above) to the plasma volume obtained by the dye injection method.

### Results.

The results of two experiments are given in Tables II and III. They indicate that the experimental variations can be limited to 5 per cent, and perhaps 3 per cent, by the method outlined.

Arnold, Carrier, Smith, and Whipple (1) have found that, during the first 4 minutes after a dog has been partially saturated with carbon monoxide through the lungs, and has begun again to breathe



normal air, the CO content of the blood does not diminish measurably, although an appreciable diminution does occur during the second 4 minutes. Our data obtained after intravenous injection of CO are similar. Besides the loss of CO from blood to muscle pigment by diffusion, one would expect excretion of the gas by the lungs to begin at maximum speed as soon as the animal respire air free from the gas, and we are at a loss to explain the

TABLE II.

Dog, weight 12.35 kilos. Injected 50 cc. of another dog's blood with 20.45 volume per cent CO = 10.22 cc. of CO.

Time from injection to drawing blood sample.	$P_{CO}$ from 5 cc. of blood at 0.5 cc. volume.	Temperature of gas at $P_{CO}$ measurement.	CO concentration in blood.	CO content of body.	Blood volume calculated by Equation 3.		CO-binding capacity of blood.	Total CO-binding capacity of body calculated by Equation 1.	Estimated fall in CO capacity of body due to drawing of blood samples.
					Total.	Per kilo.			
min.	mm.	°C.	vol. per cent	cc.	cc.	cc.	vol. per cent	cc.	cc.
3	78.8	24	0.97	10.22	1054	85	13.8	145	
	81.0	23	1.00	10.22	1022	83	13.8	141	
6	79.5	23	0.98	10.02*	1028	83	13.6	139	2.0
	81.0	22.5	1.00	10.02	1007	82	13.6	136	
10	75.0	22	0.93	9.82*	1055	85	13.2	139	4.0
	74.0	23	0.92	9.82	1066	86	13.2	140	

\* Corrected by subtracting from injected CO the amount withdrawn in preceding blood samples of 20 cc. each. This corrected value is used, in place of the total injected CO, in calculating by Equation 1 the figures in the next to the last column.

apparent lag in excretion during the first 4 minutes. Nevertheless Whipple's blood analyses and ours indicate that the lag occurs.

The decreases that occur in the CO content in the second 5 minutes of our experiments are due in part to the removal of blood samples each amounting to 2 or more per cent of the animal's total blood. These removals decrease the CO-binding capacity of the body by the amounts indicated in the last columns

of Tables II and III, and should decrease the capacity estimated from the blood CO content by the same amounts if there were no losses of CO from the circulating blood. In the experiment of Table II it seems doubtful that appreciable loss occurred in

TABLE III.

Dog, weight 14.25 kilos. Drew and defibrinated 110 cc. of this animal's own blood. Saturated a portion with CO. Reinjectd 50 cc. of blood of 19.06 volume per cent CO = 9.53 cc. of CO.

Time from injection to drawing blood sample.	$P_{CO}$ from 5 cc. of blood at 0.5 cc. volume.	Temperature of gas at $P_{CO}$ measurement.	CO concentration in blood.	CO content of body.	Blood volume calculated by Equation 3.		CO-binding capacity of blood.	Total CO-binding capacity of body calculated by Equation 1.	Total red cell volume in body calculated by Equation 2.*	Estimated fall in CO capacity of body due to drawing of blood samples.
					Total.	Per kilo.				
min.	mm.	°C.	vol. per cent	cc.	cc.	cc.	vol. per cent	cc.	cc.	cc.
3	102.0	23.0	1.26	9.53	755	52.5	14.7	111	247	0
	102.1	23.0	1.26		755	52.5		111	247	
5	102.8	22.5	1.27	9.28†	731	52.3	14.8	108	240	3.0
	100.3	27.5	1.24		749	53.6		111	247	
8	91.4	22.5	1.13	9.03†	798	56.0	14.0	112	249	5.5
10	87.3	22.5	1.08	8.80†	813	57.0	13.8	112	249	7.8

\*  $f$  of Equation 2 was found to be 2.22. The combining capacity of the defibrinated blood was  $19.06 - 1.84 = 17.22$  volume per cent. The cell volume by three hematocrits was found to be 38.1, 38.0, 38.3, average 38.2 per cent of the blood volume. Therefore  $f = \frac{38.2}{17.22} = 2.22$ .

† Corrected by subtracting from injected CO the amount withdrawn in preceding blood samples of 20 cc. each. This corrected value is used, in place of the total injected CO, in calculating by Equation 1 the figures in the next to the last column.

the 10 minutes of the experiment. In the experiment of Table III the CO capacity of the body calculated from the analyses remained constant while enough blood was drawn to reduce it by 7 per cent. Apparently therefore a loss of about 7 per cent

of the circulating CO occurred in the 10 minutes of the experiment, chiefly in the second 5.

#### SUMMARY.

A technique is described for quantitative gasometric determination of small amounts of carbon monoxide in blood.

It appears that 4 minutes after injecting carbon monoxide blood as described in this paper one can estimate the volume of the circulating red cells from the blood CO content with less than 5 per cent error due to analytical technique and CO distribution within the blood. We have not ascertained the magnitude of the possible additional error due to diffusion of CO from blood to tissue hemoglobin.

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## **MICRO METHODS FOR THE DETERMINATION OF LABILE AND TOTAL SULFUR IN PROTEINS.\***

By L. C. MAXWELL, FRITZ BISCHOFF, AND N. R. BLATHERWICK.

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)*

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In their preliminary report on the labile sulfur in insulin, Abel and Geiling (1) describe a quantitative method whereby a determination of labile sulfur may be made upon from 50 to 100 mg. of material. A nephelometric method has been developed in this laboratory requiring only 5 mg. of insulin for an analysis. Several hundred determinations have been made upon various insulins, insulin derivatives, and organic compounds containing labile sulfur. Such an extensive investigation would have been prohibitive with any method which required more than a few hundred units of insulin for a determination.

Although nephelometric methods are in general unpopular and subject to severe and just criticism, no other principle was found feasible. The method finally adopted by us was found accurate to 10 per cent for amounts of sulfur ranging from 0.1 to 0.3 mg. Below 0.1 mg. the accuracy was decreased and below 0.05 mg. the results must be interpreted as qualitative rather than quantitative. The principle of a nephelometric determination has also been adapted to total sulfur analyses of organic compounds. Since the interest in these procedures, especially the one dealing with the total sulfur, is entirely aside from the interpretation of the results obtained with insulin, the analytical methods are presented separately.

The technique embodying part of the procedure used by Abel for the determination of labile sulfur, combined with a nephelometric estimation of the sulfur as barium sulfate, was found satisfactory.

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Abel has chosen arbitrarily 0.1 N sodium carbonate and 10 per cent sodium hydroxide as the degrees of alkalinity to differentiate between more or less labile sulfur. So that our results would be comparable with theirs, the same solutions were used in liberating the sulfur. In the preliminary investigations the heating with alkali was done in an atmosphere of hydrogen. The possibility that the hydrogen might exert a reducing action upon the sulfur presented itself. In the later determinations nitrogen was substituted for hydrogen. A series of determinations was made using both hydrogen and nitrogen and the results were found to be identical in all cases. After the sample has been heated with the alkali in an atmosphere of inert gas for a specified time, it is acidified with hydrochloric acid and the liberated hydrogen sulfide swept over and oxidized in a solution of hypobromite. At this point the procedure differs from that of Abel who separated the sulfur as cadmium sulfide. Denis (2) has worked out a nephelometric method for the determination of sulfates in blood. The conditions used in her method for the formation of the barium sulfate suspension have been followed to a considerable extent. Attempts were made to dispense with the sweeping over of the liberated hydrogen sulfide into another container by separating the protein material with mercuric chloride as Denis does in her blood analysis. It was found that the oxidation with hypobromite, which by necessity precedes the mercuric chloride precipitation of the proteins, yielded decomposition products which would not completely precipitate with mercuric chloride, and a cloudy solution often resulted. It was also found that by this method some of the oxidized sulfur was lost in the protein precipitate, and that in solutions containing larger amounts of protein material the loss of sulfur was proportionately increased.

*Method for Estimation of Labile Sulfur.*

*Principle.*—The sample in weak alkali is heated in a current of nitrogen for a specified time. The solution is then acidified and the liberated hydrogen sulfide oxidized and the sulfur estimated as barium sulfate with the nephelometer.

*Procedure.*—The solution containing from 0.1 to 0.2 mg. of sulfur is placed in the test-tube A and the air displaced by a slow current of nitrogen. An equal volume of 0.2 N sodium carbonate

or 20 per cent sodium hydroxide is added through the dropping funnel to give a concentration of 0.1 N sodium carbonate or 10 per cent sodium hydroxide, depending on the type of labile sulfur to be determined. A slow current of nitrogen is bubbled through the solution while it is being heated in a boiling water bath. The heating is carried on for 30 minutes. The water bath is then filled with cold water and the test-tube A connected with a receiving tube B containing 10 to 15 cc. of dilute bromine water to which a few drops of alkali have been added. Sufficient hydrochloric acid is then added through the dropping funnel to acidify the contents of A and the liberated hydrogen sulfide is swept over into the hypobromite solution by a slow current of nitrogen. After complete removal of the hydrogen sulfide, from 20 to 30 minutes, the receiving flask is disconnected. The hypobromite solution is acidified and the bromine boiled off. After cooling and making neutral or just faintly acid to litmus, 4 cc. of a 10 per cent ammonium nitrate solution and 10 cc. of a 1 per cent barium chloride solution containing 0.5 per cent hydrochloric acid are added and the volume made to 50 cc. After standing for 15 minutes the solution is compared with a standard made to the same volume and prepared at the same time. The standard contains from 0.1 to 0.3 mg. of sulfur as potassium sulfate. To insure maximum accuracy the turbidity of the standard and the unknown should be approximately the same with a maximum variation of not over 30 per cent. A standard type nephelometer is used for the comparison. See Fig. 1.

The determination of sodium carbonate and sodium hydroxide labile sulfur may be made upon the same sample provided the difference between the two types of sulfur is greater than 0.1 mg. The preliminary heating in the carbonate determination does not appreciably affect the amount of sulfur obtained on subsequent heating with sodium hydroxide.

The modified Parr (3) method for obtaining a colloidal barium sulfate precipitate may be substituted in the above procedure. With this procedure approximately 0.1 gm. of a mixture containing equal parts of barium chloride and potassium oxalate crystals, finely powdered, is added to the faintly acid solution of standard and unknown and the flasks are shaken at intervals for 15 minutes and then compared in the nephelometer.

Due to the fact that barium sulfate is more soluble in acids than in water, it is essential, when the barium sulfate precipitation is made, that the acidity be very carefully regulated. Variations due to the solubility of the sulfate in acid are evaded by keeping the acidity low and by having the degree of acidity of the standard and the solution to be determined approximately the same.

The commercial nitrogen used in these experiments was bubbled

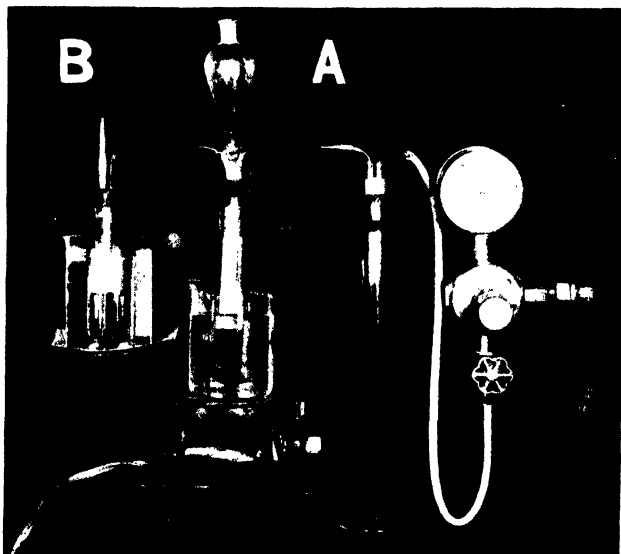


FIG. 1.

through a tower containing alkaline pyrogallol until free from oxygen. Sulfur-free tubing and reagents were used but blanks equivalent to 0.01 mg. of sulfur for the sodium hydroxide and 0.02 mg. of sulfur for the sodium carbonate were obtained. In order to obtain uniform blanks a total volume of 10 cc. of 10 per cent sodium hydroxide or 0.1 N sodium carbonate was used.

In the total sulfur determination, the conditions for precipitation of the barium sulfate worked out by Parr (3) gave the most satisfactory colloid. The high concentration of sodium chloride resulting from the peroxide fusion has a tendency to render the barium sulfate precipitate crystalline when ammonium nitrate

is used in the precipitation. It was found that the oxalic acid used by Parr in the determination of sulfur in coal gave a more colloidal barium sulfate precipitate. This procedure, modified somewhat, was later used both in the total and in the labile sulfur determinations and in our opinion gives a more stable colloidal barium sulfate precipitate than is obtainable by the Denis method.

*Method for Estimation of Total Sulfur.*

*Principle.*—In the determination of total sulfur in proteins a sodium peroxide fusion is made and the sulfur estimated as barium sulfate with the nephelometer.

TABLE I.  
*Specimen Analyses by the Micro Methods.*

Substance.	Sulfur determined.	Sulfur by other methods.
Na <sub>2</sub> S·10 H <sub>2</sub> O	0.11 mg. 0.19 "	0.10 mg. taken. 0.20 " "
Casein.	0.83 per cent, total.	0.80* per cent, total.
Cystine.	29.0 " " "	26.6 " " "
	2.0 " " labile with 10 per cent NaOH.	1.99† " " labile with 10 per cent NaOH.

\* Mathews, A. P., *Physiological chemistry*, New York, 2nd edition, 1916, 119.

† Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 423.

*Procedure.*—Sufficient sample to give 0.1 to 0.3 mg. of sulfur is placed in a nickel crucible and mixed thoroughly with from 0.5 to 1.0 gm. of pure anhydrous sodium carbonate. If the sample is in solution it should be taken to dryness in the crucible in an oven at a moderate temperature before mixing with the sodium carbonate. The contents are then moistened with a few drops of water and pure sodium peroxide added in small amounts with stirring until the mixture becomes nearly dry and quite granular. The crucible is then heated in a muffle or over a sulfur-free flame until fusion takes place, rotating it about so that any particles adhering to the side will come in contact with the oxidizing material. The heating is continued for 10 minutes after fusion takes place. The crucible is allowed to cool somewhat and the contents dissolved in



15 to 20 cc. of water and rinsed into a flask. Concentrated hydrochloric acid is added until the solution is slightly acid. It is then boiled to decompose oxidizing materials, cooled, and made slightly alkaline and filtered. The clear filtrate is made slightly acid and 4 cc. of a 10 per cent ammonium nitrate solution and 1 cc. of a 10 per cent barium chloride solution containing 5 per cent hydrochloric acid are added. A blank fusion is made simultaneously, using the same quantities of carbonate and peroxide, and it is added to the standard containing 0.1 to 0.3 mg. of sulfur as potassium sulfate. The volumes of the standard and the unknown are made up to 50 cc. and after standing for 15 minutes compared in the nephelometer.

Table I contains the results of sulfur analyses as determined by the methods described in this paper.

The Parr (3) method of barium sulfate precipitation can be advantageously used in connection with this procedure. After boiling vigorously to decompose the hydrogen peroxide and making slightly acid, the solution is cooled and approximately 0.1 gm. of the modified Parr sulfate precipitant (a mixture of equal parts of barium chloride and potassium oxalate, finely powdered) is added and the flasks shaken at intervals for 15 minutes. The volumes are then made to 50 cc. and compared with a standard prepared simultaneously. Readings are made in the nephelometer.

Various oxidation procedures were tried before adopting the standard peroxide fusion method as most suitable for incorporation in a micro method for total sulfur determination.

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## STUDIES ON INSULIN.\*

By N. R. BLATHERWICK, FRITZ BISCHOFF, L. C. MAXWELL,  
JOHN BERGER, AND MELVILLE SAHYUN.

(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara  
Cottage Hospital, Santa Barbara.)

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This laboratory has prepared large amounts of insulin for use in the Potter Metabolic Clinic during the past 4 years. As opportunity afforded, various observations concerning the properties of insulin have been made. Our findings have been reported only in a preliminary manner because we believed that a more thorough study should precede publication. In this paper we report a method for preparing insulin, methods of purification, observations on the effects of various chemical reagents upon insulin, and an extended study of the labile sulfur of insulin. We also comment upon the chemical properties of insulin.

### *Preparation of Insulin.*

The fresh pancreas of beeves and hogs are trimmed of fat and extraneous matter. They are kept on ice until a quantity has accumulated sufficient for a "run," about 30 pounds. The chilled glands are then ground to a pasty consistency in a Buffalo chopper. 20 pounds of this meat are placed in a rotary mixer together with 1 gallon of water and 100 cc. of concentrated sulfuric acid. After the mixer has run for 15 minutes, 5 gallons of 95 per cent alcohol (ethyl denatured with 10 per cent methyl) are added. The mixing is then continued to a total of 1 hour. The juice is pressed from this mixture by means of a heavy power press. At this point the concentration of alcohol should be 63 to 65 per cent and, if it is too low, enough 95 per cent alcohol is added to attain this concentration. The hydrogen ion concentration is about

\* This work was supported in part by a special insulin grant from the Carnegie Corporation of New York.

pH 2.5 to 3.0. The solution is next filtered. About 50 cc. of sodium hydroxide (sp. gr. of 1.50) are added to each 10 gallons of filtrate. It is important to make this partial neutralization rather exact in order to secure rapid filtration from the fat later in the process. The extract is concentrated *in vacuo* to about  $\frac{1}{10}$  its volume. The temperature of this residue is about 45°C. upon removal from the water bath which is regulated to maintain a temperature of 80°C. The concentrate is filtered immediately through double folded filter papers. The filter papers containing the dark brown, fatty precipitate are extracted with enough water to cover and filtered the next day. The active material thus recovered is worth the labor involved. This filtrate is added to the main extract.

The clear aqueous filtrate is saturated by adding 22 per cent of commercial sodium chloride, which throws out a dark precipitate containing the active principle. After standing  $\frac{1}{2}$  hour, the underlying solution is syphoned off. The salt precipitate is dissolved in the original volume of water and without changing the reaction again saturated with sodium chloride. The precipitate containing the active material rises to the surface and the salt water is again syphoned off and discarded. The second saturation with sodium chloride seems to remove most of the contaminating, inactive protein which precipitates at a pH of 7.0. Further precipitations with sodium chloride do not effect a greater purification. This moist salt precipitate is dissolved in 95 per cent alcohol, using about 500 cc. for each 96 quarts of original alcoholic extract. The alcoholic extract is filtered or centrifuged to remove some dark insoluble matter which is largely salt. By adding the clear solution to 2 volumes of ether, a precipitate containing all the active material is thrown down. After 24 hours, the supernatant alcohol-ether mixture is drawn off and the ether is removed from the precipitate on a vacuum line.

This precipitate is dissolved in sufficient water, about 1 liter for each 50 quarts of original alcoholic extract. It usually holds enough acid to insure solution. Sodium hydroxide is added to the clear solution until the isoelectric point is reached, pH 4.4 to 5.0. After standing overnight, the liquid is centrifuged and the precipitate is redissolved in approximately  $\frac{1}{2}$  the volume of water and the necessary amount of hydrochloric acid. After

the third isoelectric precipitation, the precipitate is dissolved in double distilled, sterile water and made acid with hydrochloric acid to a pH of 3.8 to 4.0.

The potency is then tested on rabbits, after which enough sterile water is added to obtain the desired strength. Phenol is added to a concentration of 0.2 per cent and enough sodium chloride to make the solution isotonic. The liquid is drawn through a Mandler filter and bottled. Bacteriological tests are made to insure sterility. Such preparations appear to keep indefinitely at room temperature without loss of potency.

The yields of crude insulin by this method vary from 1800 to 2500 clinical units per kilo of pancreas.<sup>1</sup> After the third isoelectric precipitation from water, from 1000 to 1400 units with a nitrogen content of about 0.006 mg. per unit are obtained from 1 kilo of material. Small amounts of the insulin remaining in the filtrates from the isoelectric precipitates may be recovered by salting, taking up in water, and precipitating at the isoelectric points. The greater part of this insulin is, however, very difficult to separate in any degree of purity. A rather satisfactory way of doing so is described in a later section under phenol treatment. The method of preparation outlined is a combination of those described by Scott and Best (1) and by Somogyi, Doisy, and Shaffer (2). Sodium chloride instead of ammonium sulfate is used as the precipitant. The use of sodium chloride seems preferable, not only because less salt is required, but because it is convenient to use when one desires to follow the nitrogen distribution of the various fractions. Also there is no danger of introducing sulfates when a study of the sulfur content of insulin is being made.

### *Purification of Insulin.*

(a) *Fractional Precipitation with Alcohol.*—A less pure insulin is obtained when but one salt precipitation is made in the method

<sup>1</sup> The unit referred to in this paper is the clinical unit. Lilly's "iletin" U 80,80390-758690 was used as the standard. Our preparations were assayed by the method developed in this laboratory. Sahyun, M., and Blatherwick, N. R., *Am. J. Physiol.*, 1926, lxxvi, 677.

described above. This crude insulin, when dissolved in alcohol, precipitated with ether, and precipitated once from water at the isoelectric point, contains at least two substances. These can be rather completely separated by utilizing their different solu-

TABLE I.  
*Fractional Precipitation of Insulin with Alcohol.*

Experiment No.	Material.	Units.	Nitrogen per unit. <i>mg.</i>
I	Before alcohol separation.	28,600	0.0045
	Insoluble, 86 per cent alcohol.	14,430	
	Soluble, 86 " " "	16,600	
II	Insoluble, 86 per cent alcohol.	16,500	
	Soluble, 86 " " "	6,300	
III	Insoluble, 77 per cent alcohol.	7,500	
	Soluble, 77 " " "	16,600	
IV	Insoluble, 77 per cent alcohol.	15,000	0.018
	" 87 " " "	35,000	0.0052
	" 92 " " "	35,000	0.0055
V	Insoluble, 75 per cent alcohol.	10,000	0.0496
	Soluble, 75 " " "	166,000	0.0051
VI	Insoluble, 75 per cent alcohol, pH 6.	10,000	0.059
	" 75 " " " " 4.6.	9,000	0.050
	Soluble, 75 " " "	83,000	0.0058
	Isoelectric precipitate of above.	83,000	0.0040
VII	Solution of ether precipitate.	355,000	0.014
	Isoelectric precipitate of above.	286,000	0.011
	Insoluble, 75 per cent alcohol.	15,500	0.019
	Soluble, 75 " " "	200,000	0.0048
	Isoelectric precipitate of above.	169,000	0.0038

bilities in alcohol. If one dissolves the above material in a minimum amount of water and sodium hydroxide, adds alcohol to a concentration of about 75 per cent (hydrometer), and adds hydrochloric acid to a pH of about 6.6, a dirty, meaty looking precipitate is formed. This material is found to be relatively

impure and carries most of the brown-red color of the original solution. Upon the addition of absolute alcohol to the filtrate from this precipitate, in amount to make a concentration of 92 per cent alcohol, and upon adjusting to a slightly more acid reaction, a flocculent, white precipitate forms. This material is much purer than the other fraction and carries most of the activity. It has a pale, straw color in dilute acid solution at a concentration of 100 units per cc. Great care must be observed in adjusting the reaction and in employing the correct concentrations of alcohol. The purpose of the separation at 75 per cent concentration is to avoid the isoelectric point of the purer material. The impurities may also be eliminated by adjusting to the acid side of the isoelectric point. If the concentration of alcohol is over 75 per cent, there is danger of losing some of the purer product in the precipitate which forms. The effect of using higher concentrations of alcohol is shown in Experiments I and II of Table I. Typical results of the fractionation are shown in the table. It is the best method we have found for purifying insulin. These observations confirm and extend the findings of Somogyi, Doisy, and Shaffer (2).

(b) *Dialysis*.—The material used in these experiments was extracted according to the original Collip method. After concentration and removal of fat with ether, the aqueous solution was saturated with sodium chloride. This precipitate was dissolved in alcohol and again precipitated by adding to ether. The aqueous solution of the ether precipitate was used for the dialysis experiments. Rather thin collodion membranes were used. Before dialysis the solutions were adjusted to barely the alkaline side of the isoelectric point. Dialysis was continued for about 5 hours, with three changes of distilled water. The first experiment showed that 4 per cent of the active material dialyzed. 63 per cent of the potency was found in the isoelectric precipitate of the residue and 33 per cent remained in the filtrate. The biuret reaction of the dialysate, after concentration, was a distinct pink in contrast to the violet color of the isoelectric material.

The second experiment made use of similar material. During dialysis a precipitate formed within the sac. This precipitate was removed and kept separate from the isoelectric material

obtained from the residual fluid. The first material proved to be less pure than the other and was more difficult to purify by

TABLE II.  
*Isoelectric Precipitation of Insulin After Dialysis.*

Experiment No.	Material.	Units.	Nitrogen per unit.
			mg.
I	Residual material after isoelectric precipitation.	21,000	0.0060
	Isoelectric precipitate of above.	21,000	0.0043
	" " " "	21,000	0.0035
	" " " "	21,000	0.0031
II	Residual isoelectric material after removal of precipitate which formed during dialysis.	45,000	0.0065
	Isoelectric precipitate of above.	36,000	0.0050
	" " " "	35,000	0.0045
	" " " "	35,000	0.0036
	Material which precipitated during dialysis.	16,500	0.0140
	Isoelectric precipitate of above.	15,000	0.0103
	" " " "	15,000	0.0061
	" " " "	15,000	0.0052
	" " " "	12,500	0.0040
III	Residual isoelectric material after removal of precipitate which formed during dialysis.	12,500	0.0084
	Isoelectric precipitate of above.	12,500	0.0046
	" " " "	12,500	0.0042
	Material which precipitated during dialysis.	25,000	0.010
	Isoelectric precipitate of above.	22,000	0.0066
	" " " "	22,000	0.0053
	" " " "	22,000	0.0049
	" " " "	20,000	0.0042
	" " " "	20,000	0.0036

the isoelectric method. The isoelectric point of these preparations when purified was about pH 6.0 (colorimetric). The solutions

were colorless in concentrations of approximately 70 units per cc. The experimental results are shown in Table II.

Dialysis enables a separation of two fractions to be made, one of which is purer and is easier to purify further. Probably the chief advantage gained is a sharper precipitation at the isoelectric point, due to the low concentration of inorganic salts. Loss of potency is therefore not so great.

(c) *Heating*.—Insulin is precipitable by heating aqueous solutions under certain limited conditions. The pH must be about 4.0, a slight deviation causing either partial or no precipitation; the concentration must be more than 50 units per cc., and the sodium chloride content must be about 1 per cent.

The solution is heated in an air or water bath at a temperature of 80°C. for a period of from 60 to 90 minutes. A precipitate usually begins to form after 15 to 20 minutes heating. It has an entirely different appearance than the ordinary isoelectric precipitate. It forms in a more or less finely divided state making separation by the centrifuge very difficult. Oftentimes it tends to settle as a gel. The precipitate formed under these conditions is acid-insoluble but becomes soluble in acid after having been dissolved in dilute alkali. The material obtained is probably a denatured product which then coagulates under the particular experimental conditions. Sometimes a very great purification is effected, in other cases little, if any, change is brought about. The danger of immediate loss in potency due to heating for the period specified is practically negligible. However, aqueous solutions of these heat precipitates appear to be much less stable under ordinary conditions of light and temperature than the material which has not been so treated. An example will suffice to show the results of this treatment. 2100 units of insulin in a volume of 20 cc. were treated as above. All of the active substance was recovered in the heat precipitate. The nitrogen content was 0.0085 mg. per unit before, and 0.0035 mg. after heating.

(d) *Hydrochloride Purification*.—There are several references in the literature to the precipitation of insulin by high concentrations of acid. Maloney and Findlay (3) found that insulin was precipitated by 5 N acid with loss of potency. We have been



able to purify insulin in several instances by using the minimum amount of hydrochloric acid required for precipitation. The greater proportion of the potent material is precipitated with some protein. About 4 per cent of hydrochloric acid is required to bring about complete precipitation. The distribution of the nitrogen is illustrated in Table III.

Experiments I, II, and III were performed in test-tubes. Concentrated hydrochloric acid was added, drop by drop, until no more precipitate formed. The precipitate was separated immediately by the centrifuge. In Experiments I and II there was no loss of potency. In Experiment II practically all of the potency was found in the precipitate with an elimina-

TABLE III.  
*Precipitation of Insulin with Hydrochloric Acid.*

Experiment No.	HCl	Original.		HCl precipitate.		HCl filtrate.	
		Units.	N per unit.	Units.	N per unit.	Units.	N per unit.
	<i>per cent</i>		<i>mg.</i>		<i>mg.</i>		<i>mg.</i>
I		1,000	0.0055	850	0.0044	140	0.011
II		1,000	0.0063	950	0.0042	50	0.048
III		1,100	0.028	550	0.043	80	0.12
IV	4.0	58,000	0.0064	55,600	0.0056	2,000	0.027
V	3.2	55,600	0.0056	40,000	0.0071	1,500	0.022
VI	2.1	40,000		22,000	0.012		
	4.2			5,500	0.010		
VII	2.7	16,500	0.037	3,500	0.054		
	5.4			1,500	0.100		

tion of one-third of the original nitrogen. Experiments IV, V, VI, and VII were performed with larger amounts of insulin, so that the exact acidity might be determined. In these experiments the solution was kept in an ice bath while the precipitation was made. It will be noted that in Experiments III and VII, where the original insulin was quite impure, a large loss of potency resulted. This is contrary to the usual behavior of insulin and may have been accidental. Experiments V and VI were a continuation of Experiment IV. In Experiment IV, a purification with no loss of potency resulted. When the hydrochloric acid precipitation was repeated, 30 per cent of the potency was lost. We do not believe that the degree of purification sometimes resulting from a hydrochloric acid precipitation overbalances the attendant risk of destroying the active substance.

(e) *Precipitation with Pyridine.*—The precipitation of insulin by pyridine resembles any other precipitation at the isoelectric

point. Pyridine is convenient to use because of its weak alkalinity. A considerable excess of the reagent must be added before solution of the precipitate occurs. In one instance a 3 per cent solution of pyridine in water was required to dissolve a sample of insulin. This insulin was soluble in 82 per cent pyridine, but it was insoluble in greater concentration. We have had similar results with other organic bases, such as aniline and benzidine. In the case of aniline, which is a much stronger base than pyridine, a slight excess will redissolve the precipitate. The pH of the filtrates from the pyridine precipitates is the same as the isoelectric point of the insulin used. It has been repeatedly demonstrated in this laboratory that after several precipitations at the isoelectric point, no further purification can be obtained by this procedure. The same is true with the pyridine precipitation. 25,000 units of insulin, with a nitrogen content of 0.038 mg. per unit, were subjected to five pyridine precipitations and to two isoelectric precipitations using sodium hydroxide to remove the pyridine nitrogen. 16,000 units with a nitrogen content of 0.032 mg. per unit were obtained. In another instance the nitrogen was reduced from 0.016 to 0.014 mg. per unit by two pyridine precipitations. The degree of purification is the same in both cases.

(f) *Phenol Treatment*.—Abel and Geiling (4) have made use of the fractional solubility of insulin in phenol to effect purification. They found that the phenol-insoluble residue contained very little if any potency. In using their method upon impure insulin residues, an additional separation of impurities may be obtained by somewhat altering the conditions of precipitating the insulin when the phenol is added to the water. When the phenolic solution is added to the minimum amount of water (15 parts water to 1 part phenol), a colloidal solution and a precipitate are formed. The precipitate is centrifuged off. The colloid contains more than half the potency in a purified form. The colloidal solution can only be separated in part by the Sharples supercentrifuge. This material is recovered from the phenolic liquid by saturating with salt. The following example illustrates the treatment. 71,000 units of insulin, with a nitrogen content of 0.11 mg. per unit, were dissolved in 59 gm. of phenol. In

this instance the substance was completely phenol-soluble. The colloidal solution, obtained by adding to water and centrifuging off the sticky precipitate, was run through the supercentrifuge. 25,000 units, with a nitrogen content of 0.050 mg. per unit, were found in colloidal solution. 22,000 units, with a nitrogen content of 0.028 mg. per unit, remained in the precipitate retained in the supercentrifuge. The colloidal solution of insulin, mentioned above, is not always found.

The phenol treatment has been used in recovering the insulin from the impure residues of the manufacturing process. The insulin remaining in these solutions is salted out. Attempts at recovering insulin from this dried precipitate, which contains from  $\frac{1}{2}$  to 1 unit per mg., were unsuccessful until the phenol technique was applied. A phenol separation, followed by a fractional alcohol precipitation, gives a good insulin, but the yields are low. 12,000 units, with a nitrogen content of 0.0063 mg. per unit, were obtained from 71,000 units having a nitrogen content of 0.11 mg. per unit. The following procedure was used.

The dried salt precipitate is powdered. 1 part by weight of powder and 2 parts of absolute phenol are heated together for 1 hour in a water bath at a temperature not to exceed 60°C. Most of the powder dissolves. The solution is centrifuged and the clear supernatant liquid is added to exactly 30 parts of water. In doing this it is well to have the water under agitation and then add the phenol drop by drop. The precipitate which forms is separated by centrifuging. It is discarded. The supernatant liquid is salted, whereupon a tarry residue separates. This residue is subjected to two isoelectric purifications. Precipitates may be found at more than one pH. These precipitates are combined. The final purification is the fractional alcohol precipitation described above. The precipitate which forms at a concentration of 75 per cent alcohol is very impure and is discarded. The soluble fraction contains the purified insulin which may be separated completely by the addition of more absolute alcohol and ether.

#### *Action Of Various Chemical Reagents upon Insulin.*

(a) *Hydrogen Sulfide.*—Murlin (5) was able to inactivate insulin by exposing it to hydrogen sulfide and to reactivate it by subsequent exposure to air or oxygen. This we were unable to do. A very impure insulin analyzing 1 unit per mg. was used in the first experiment and no inactivation was found. Exposures of a purer insulin, assaying 0.0055 mg. N per unit, resulted in the

complete and irreversible inactivation of the insulin. These preliminary experiments indicated the presence of a protective

TABLE IV.  
*Effect of Hydrogen Sulfide in Solution on Insulin.*

Nitro- gen per unit.	Medium.	Concentration.	pH	Time.	Original poten- cy.	Recoy- ered poten- cy.
mg.				hrs.	units	units
0.0055	0.001 N H <sub>2</sub> S.	100 units in 50 cc.	6.2	24	100	0
0.0055	0.001 " "	100 " " 50 "	6.0	24	100	0
0.0055	0.010 " Congo red.	100 " " 50 "		24	100	100
0.0055	0.010 " H <sub>2</sub> S. 0.010 " Congo red.	100 " " 50 "	*	24	100	25
0.0055	0.005 " H <sub>2</sub> S. 0.010 " Congo red.	100 " " 50 "		84	100	55
0.0055	0.001 " H <sub>2</sub> S.	200 " " 50 "		48	200	100
0.0055	0.005 " " 0.5 per cent casein.	200 " " 50 "		48	200	165
0.0055	0.005 N H <sub>2</sub> S.	50 " " 50 "		48	50	12
0.016	0.001 " "	100 " " 50 "	7.8	24	100	100
0.016	0.001 " "	100 " " 50 "	5.6	72	100	75
0.070	0.001 " "	200 " " 50 "	8.0 to 4.5	24	200	180
0.070	0.010 " "	200 " " 50 "	4.0	24	200	100

\* The pH of the Congo red solutions was adjusted so that the dye was at the transition between the blue and red forms.

substance in the impure insulin and they have led to a study of the effect of the purity upon the stability.

*Action of Sulfide in Aqueous Solution.*—We used insulins of

varying degrees of purity, analyzing respectively 0.0055, 0.016, and 0.070 mg. of nitrogen per unit. In 0.001 N sulfide solution adjusted to a slightly acid pH, the insulin, analyzing 0.0055 mg. of N per unit, was completely inactivated in 24 hours. Under the same conditions, the 0.016 mg. of N insulin retained three-fourths of its activity after 72 hours, and the 0.070 mg. of N insulin, 90 per cent of its activity after 24 hours. In another experiment similar results were obtained with a 0.01 N solution but the odor of sulfide was strong after 72 hours. This invalidated the possibility that the impurities of the insulin functioned by destroying the hydrogen sulfide. The calculated amount of hydrogen sulfide in solution required to inactivate completely a

TABLE V.  
*Effect of Hydrogen Sulfide Gas on Dry Insulin.*

Experiment No.	Nitrogen per unit.	Time.	Treatment.	Potency in units per mg.	
				Before.	After.
I	mg.	hrs.			
I	0.16	24	Dry H <sub>2</sub> S atmosphere.	0.9	0.9
II	0.0055	48	" " "	22.0	15.0
III			Material treated with H <sub>2</sub> S in Experiment II. Exposed to air current for 24 hours.	15.0	14.7
IV	0.0033	24	Dry H <sub>2</sub> S atmosphere.	39.0	16.0
V			Material treated with H <sub>2</sub> S in Experiment IV. Exposed to air current.	16.0	12.0

given amount of highly purified insulin is 0.7 mg. of sulfide for 1.5 mg. of insulin. 50 cc. of 0.001 N sulfide solution will completely inactivate 100 units of this insulin. If 200 units are tested, only half will be inactivated.

*Action of Hydrogen Sulfide Gas on Dry Insulin.*—Three samples of insulin which analyzed 0.16, 0.0066, and 0.0033 mg. of nitrogen per unit were used. Before being brought into contact with the powdered insulin, the hydrogen sulfide was dried by passing it through calcium chloride. A steady stream of the gas was conducted over the insulin powder for an hour. The container was then stoppered so that the insulin remained in an atmosphere of hydrogen sulfide. Under this treatment the very impure

insulin lost none, the fairly pure insulin lost one-fourth, and the highly purified insulin over one-half of its activity. Exposure to air for days did not reactivate the powders which had been partly inactivated by the hydrogen sulfide. An interesting observation was made. After the highly purified insulin had been subjected to the stream of hydrogen sulfide, the stopper of the container was accidentally opened, diluting the sulfide atmosphere with air. No inactivation resulted. On repeating the experiment without the introduction of air, over half the potency was destroyed. This indicates that the inactivation is preceded by an adsorption and that it is necessary not only to remove the adsorbed air from the adsorbing centers, but to keep it away while the adsorbed hydrogen sulfide is functioning in its destruction of the active group.

Since the experiments both in gas-solid phase and solution demonstrated the protective action of the impurities in the insulin, an attempt was made to find an artificial protective agent, which, when added to a very pure insulin, would inhibit the destruction of the insulin by hydrogen sulfide. The substantive dye, Congo red, was found to have this property. Congo red has several properties in common with the proteins; particularly its high adsorbing powers, the bulk of its molecule, its amphoteric nature, containing the basic amino and acidic phenolic and sulfonic acid groups, and its colloidal state of aggregation in alkaline solution. The highly purified insulin is completely inactivated by 0.001 N sulfide solution. In the presence of 0.01 N Congo red solution less than half the activity was destroyed by 0.005 N sulfide solution, 5 times the amount which would have normally destroyed it. Allen and Murlin (6) report that the biuret-free preparations they obtain from pancreas perfusates are extremely unstable, the activity being lost spontaneously within a week and very often within a few days. The chemical study of these highly purified insulins is rendered even more difficult than was first anticipated because of the great instability of the purified insulin. The results of our experiments with Congo red suggest the possibility of stabilizing such insulins by the addition of colloids of known constitution, a procedure which may be likened to the protection of amino groups by acetylation.

The addition of a foreign protein, such as casein, was also found to exert a protecting influence.

From the foregoing experiments, we may conclude that the inactivation of insulin by hydrogen sulfide is dependent upon the impurities associated with the insulin, very pure insulin being entirely inactivated and crude insulin being unaffected. With the insulins used in this laboratory, the inactivation was irreversible. From this instance as well as from others discussed in this paper, we do not intend to infer that insulins obtained by other workers will not react differently. The addition of a protective colloid to a purified insulin increased its stability to the destructive action of hydrogen sulfide.

*Experimental.*—The insulin, analyzing 0.0055 mg. of N per unit, was prepared by the routine procedure of this laboratory. That analyzing 0.016 mg. of N per unit was made, using Abel's (4) phenol, pyridine purification upon impure insulin residues. The very impure insulin was the "insulase" of Armour and Company, which is no longer on the market. The dry insulin, with a potency of 39 units to the mg., was prepared by an isoelectric precipitation of the insulin analyzing 0.0055 mg. of N per unit.

(b) *Cyanide.*—Insulin gives a negative test with nitroprusside solution, showing the absence of mercaptan groups. However, when it is treated with cyanide and then subjected to nitroprusside, a very distinct, positive test results. This confirms the observation of Scott (7) and others, that insulin contains cystine. The function of the cyanide (Walker (8)) is to reduce the disulfide cystine sulfur to the mercaptide cysteine linkage.<sup>2</sup> 4 mg. of insulin, containing 0.0053 mg. of nitrogen per unit, were used for a test.

Since cyanide attacks the insulin complex and reduces the cystine, it was interesting to determine what effect such treat-

<sup>2</sup> It has been found advantageous to modify Walker's procedure. He adds the nitroprusside first, then the cyanide. We found the sensitivity of the test was increased by reversing the order. The excess of cyanide apparently reacts with the nitroprusside, as is shown by the disappearance of the normal yellow color of the dilute nitroprusside, necessitating the addition of an excess of the latter reagent. When the cyanide is added after the nitroprusside, as Walker directs, an inappropriate balance between nitroprusside and cyanide may result, and a negative test is obtained in the presence of cystine.

ment would have upon the potency. It was found that insulin which had been exposed to the action of cyanide for 18 hours was completely inactivated. When the insulin was salted out and separated from the cyanide solution by centrifugation, it gave a positive nitroprusside test without the addition of cyanide to the solution. The experiment was repeated, exposing the insulin to the action of cyanide for only 15 minutes, when it was separated from the cyanide solution by an isoelectric precipitation. 30 per cent of the original potency was recovered. By reducing the concentration of the cyanide, 60 per cent of the potency was recovered. The insulin, which was completely inactivated by long contact with cyanide, was partly acid-insoluble. The results of these experiments indicate that the cyanide reacts with the insulin complex in more than one way; the reduction of the cystine to cysteine takes place instantly, the destruction of the groups connected with the potency more slowly, and the denaturing of the protein to an acid-insoluble form very gradually.

*Experimental.*—0.1 gm. of sodium cyanide in 1 cc. of water was added to 1000 units of insulin, analyzing 0.0055 mg. of N per unit, in 5 cc. of water. The pH of the solution was 7.0. After 15 minutes the solution was made faintly acid, saturated with sodium chloride, and centrifuged. The precipitate was dissolved in 10 cc. of water. 1 cc. gave a positive nitroprusside test without the addition of cyanide. 350 units were recovered. The experiment was repeated, subjecting the insulin to the influence of the cyanide for 18 hours. No potency was left. In a third experiment 0.1 gm. of sodium cyanide was added to 90 units of insulin in 50 cc. of water. After 15 minutes the solution was made acid and saturated with salt. 50 units of insulin were recovered. 50 units will give the nitroprusside test, but the test is not sensitive to smaller amounts. The possibility that the alkalinity of the sodium cyanide caused the destruction of the potency must be considered. A sample of this insulin dissolved in 0.1 N sodium carbonate lost three-fourths of its potency in 90 hours. This was a greater degree of alkalinity than obtained in the experiments with cyanide.

(c) *Nitrite.*—Scott (7) has studied the effect of nitrous acid upon insulin. Under the conditions of his experiments in which a very large excess of nitrous acid was used (1 gm. of nitrite for 20 mg. of insulin), inactivation resulted. He used acetic acid as his solution medium. By using very dilute aqueous nitrous



acid solutions, we have been able to affect the insulin complex without destroying the potency. Not only the amount but the concentration of the nitrous acid is an important factor in regulating its destructive effect upon the potency. Insulin is stable in a 0.002 N nitrous acid solution. In a 0.05 N solution, from 15 to 30 per cent of the potency is destroyed and the product becomes nearly acid-insoluble. The biuret test remains positive. Nitrous acid has a marked effect upon the labile sulfur of the insulin. This will be considered in that section of the paper devoted to labile sulfur. In concentrated solution nitrous acid is unstable, decomposing to various oxides of nitrogen. The inactivation of insulin by concentrated nitrous acid solutions is no doubt due to the oxidizing effect of these decomposition products. From a structural point of view, it is important to know that the active insulin group does not react with nitrous acid itself.

*Experimental.*—100 units of insulin, having a nitrogen content of 0.0033 mg. per unit, 1 drop of 10 per cent hydrochloric acid, and 3 mg. of sodium nitrite were made up to 25 cc. and kept below 5°C. for 12 hours. The pH of the mixture was 3.4. The solution was used for rabbit tests without further adjustment. None of the potency was destroyed.

1000 units of insulin, having a nitrogen content of 0.0055 mg. per unit, 2 drops of 10 per cent hydrochloric acid, and 30 mg. of sodium nitrite were permitted to react for 4 hours at a dilution of 10 cc. A yellow precipitate formed. The insulin was salted out, taken up in 25 cc. of acidulated water, to which a drop of concentrated ammonia was added to destroy any occluded nitrous acid. 30 per cent of the potency was destroyed. This preparation was used for labile sulfur analyses. Hence the precaution to remove all traces of nitrous acid.

The conditions of the above experiment were repeated, but the excess nitrous acid was removed after about an hour. Only 15 per cent of the potency was destroyed. 80 units of this treated material gave a strong biuret test. The solution gave a negative test with starch-iodide solution after separation from the excess nitrite. The major portion of the insulin was acid-insoluble.

(d) *Diazo Reaction.*—Shonle and Waldo (9) found that a highly purified insulin, prepared by dialysis and containing 0.002 mg. of nitrogen per unit, gave a positive Pauly (Ehrlich) diazo test with an intensity per unit proportionate to the original preparation. Scott (7) reports a positive Pauly test on insulin analyzing

0.006 mg. of nitrogen per unit. This insulin analyzed 10 per cent tyrosine by the Folin-Looney method. Since the test is characteristic for tyrosine and histidine, and for proteins containing these amino acids, it was interesting to determine whether insulin, which had been coupled with a diazo component—not necessarily the diazobenzenesulfonic acid of Ehrlich—would retain its potency. If the physiological activity of insulin is characteristic of some peculiar chemical grouping (structure), then the addition of a group such as the azo component to another part of the molecule should not affect this property. If the physiological activity of the insulin complex is not solely dependent upon some specific atomic grouping, but requires specific conditions for adsorption, or osmosis, or some other more physical property, then the addition of another component to the active molecule might so change the physical properties of the entity as to render it physiologically inactive. Inactivation of the insulin molecule would also result if the active chemical group were destroyed by the diazo compound, either by combining with it, or through an oxidation or reduction reaction.

Since the Pauly diazo test, as ordinarily carried out, would be subject in this instance to many side reactions, the conditions of experimentation were modified to eliminate as many of these influences as possible. Insulin is very sensitive to alkali. In the test as given by Mathews the mixture should be made distinctly alkaline with ammonium hydroxide or sodium carbonate. We found that the coupling takes place in very faintly alkaline solution. It was therefore unnecessary to make the insulin strongly alkaline and thus risk the destruction of most of the potency. A large excess of nitrous acid is used in diazotizing the sulfanilic acid in the Pauly test. It has been shown that concentrated nitrous acid greatly decreases the activity of insulin. In these experiments the minimum amount of nitrite (105 per cent of that required by theory) was therefore used. The coupling was carried out in ice water to eliminate the side reactions caused by heat.

Our experiments with diazo compounds have led to the following conclusions. At an acid pH insulin is stable to dilute solutions of diazonium salts. More concentrated solutions destroy

some potency. The same results were obtained with nitrous acid. In faintly alkaline solution coupling takes place, and the potency is destroyed. The amount of diazo required to destroy the insulin is considerably less than the theoretical amount required to couple with the histidine and tyrosine. Thus, 2 cc. of 0.002 N diazo solution destroyed 85 per cent of an original 200 units of insulin. On the basis of Scott's figures for the histidine and tyrosine content of insulin, 6 cc. would have been required for a complete coupling. In this inactivation, a considerable portion of the diazo was consumed in coupling, as shown by the development of a decided coloration. Although not a limiting value, because of the fact that the inactivation and coupling take place simultaneously, the value nevertheless indicates that a very small per cent of the insulin complex constitutes the active insulin group. The alkaline azo coupling completely destroys the labile sulfur. This will be considered in the section devoted to that subject.

In making a colorimetric comparison between diazotized insulin and casein, values for tyrosine and histidine which approximate those of other workers were obtained. Insulin developed a color of 1.6 times the intensity that an equal weight of casein gave. The shade of the color was exactly the same, indicating that the distribution between histidine and tyrosine in insulin and casein is approximately the same. Scott reports 10 per cent tyrosine and 5 per cent histidine. We obtained 14 per cent tyrosine and 8.5 per cent histidine colorimetrically, and 17 per cent tyrosine by the Folin-Looney method. The insulin azo compound is acid-insoluble and alkali-soluble when the azo component contains a sulfonic acid group, like diazobenzenesulfonic acid. The azo compound with tetrazodiphenyl (benzidine) is neither acid- nor alkali-soluble.

*Experimental.*—A few of the more important experiments are recorded.

5 cc. of a diazobenzene solution, made by adding 0.1 cc. of N nitrite solution to 0.5 gm. of sulfanilic acid and 1 cc. of hydrochloric acid in 100 cc. of ice water, were added to 2000 units of insulin, having a nitrogen content of 0.0055 mg. per unit. After 10 minutes the solution was saturated with salt and the insulin separated from the diazo liquor by the centrifuge. It was made to 25 cc. volume with dilute alkali and then reacidified. 75 per cent of the potency was recovered. After making alkaline, it became partly

acid-insoluble and developed a slight yellow color. This was probably due to a small amount of occluded diazo compound. The preparation was used for the labile sulfur analyses.

1000 units of the same insulin used in the above experiment were treated with an excess of diazobenzenesulfonic acid in sodium bicarbonate solution for 1 hour in an ice bath. The solution was then made acid and saturated with salt, so that the insulin azo compound might be centrifuged. Although acid-insoluble, this compound is so finely divided when first formed that it will not separate completely without the addition of salt. The potency was completely destroyed. Doses representing 20 original units failed to lower the blood sugar of rabbits.

A 0.002 N diazobenzenesulfonic acid solution was made by adding 120 per cent of the theory of required nitrite to the theoretical amount of sulfanilic acid and dilute hydrochloric acid in a volumetric flask cooled with ice. The solution was placed in the ice box for 4 hours to insure a complete diazotization. 1 and 2 cc. respectively of this diazo solution were added to 200 units of insulin in 5 cc. of phosphate buffer having a pH of 6.8. The solutions were made alkaline for 30 seconds by adding a drop of sodium hydroxide. They were then reacidified. The final dilution was 25 cc. The sample treated with 2 cc. of diazo solution lost over 85 per cent of its potency. 1 cc. of diazo solution destroyed about 75 per cent of the potency. Both samples were highly colored in alkaline solution.

Solutions of 0.0038 gm. of insulin, representing 0.0055 mg. of N per unit, and 0.0060 gm. of casein were placed in 100 cc. volumetric flasks with 1 gm. each of sodium carbonate. The volume was made up to about 75 cc. and 0.25 gm. of diazotized sulfanilic acid was added to each. They were diluted to the mark and placed on ice overnight. The insulin had just twice the color intensity of the casein solution.

(e) *Iodine*.—Brand and Sandberg (10) have suggested a possible iodometric titration of insulin. Their results seem to indicate that if the insulin preparations are of sufficient purity, the number of units can be established iodometrically by using a neutral, buffered, iodine-potassium iodide solution. They used the standard insulin preparations of Lilly, Toronto, and Squibb and found a surprisingly close agreement between cc. of iodine absorbed and number of units represented. We have performed their titration and a more rapid modification on several of our insulin materials prepared or purified in various ways. These results are summarized in Table VI. While there is a very close agreement for seven of the nine samples represented, two of them, Insulin IV and resin from Insulin X, although representing the same degree of purity, indicate a much lower capacity

for iodine absorption. When Insulin IV was one-half destroyed by exposure to ultra-violet light, it still gave the same iodine value. These results indicated that the iodine reaction in part involved groups of the insulin complex other than the active insulin group. To establish this point, the minimum amount

TABLE VI.  
*Amounts of 0.005 N Iodine Required to Saturate 1 Unit of Insulin.*

Insulin.	Nitro- gen per unit.	pH 4.5 2 hrs. at 37°C.	pH 4.5 16 hrs. at 37°C.	pH 10.0 5 min. in ccld.	pH 6.8 16 hrs. at 37°C.
	mg.	cc.	cc.	cc.	cc.
II, dialysis.....	0.0069	0.011	0.021	0.077	0.054
III, alcohol purification.....	0.0043	0.0085	0.019	0.073	0.059
IV " ".....	0.0055	0.013	0.016	0.062	0.046
IV, half destroyed by ultra-violet light.				0.062	
HCl purified.....	0.010			0.078	0.060
75 per cent alcohol-insoluble.....	0.012			0.073	
Iletin 80390-758690.....	0.0063			0.058	0.053
X, resin.....	0.0043			0.033	0.031
X, flocculent.....	0.0056			0.070	0.057

TABLE VII.  
*Inactivation of Insulin with Iodine.*

Experiment No.	Reaction.	0.005 N iodine.	Units recovered.
	pH	cc.	per cent
I	8.0	9.3	0
II	8.0	3.7	0
III	8.0	1.9	10
IV	6.8	3.7	0
V	6.8	0.93	25

200 units of insulin were used in each experiment. 1 unit weighed 0.038 mg. The following amounts of iodine were required to saturate 1 unit of insulin: at a pH of 6.8, 0.0295 mg; at a pH of 8.0, 0.0393 mg. At a pH of 8.0, 0.005 mg. were required to inactivate 1 unit of insulin.

of iodine to inactivate a definite number of units was determined. Only one-seventh the iodine that was absorbed was required to inactivate. The exact figures are given in Table VII. Brand and Sandberg found that the iodine destroyed the lead-blackening sulfur of insulin. We have found that about 50 per cent of the

labile sulfur in insulin has no connection with the potency. Iodine reacts with cystine and such phenolic bodies as tyrosine. Different insulin preparations contain varying amounts of these amino acids. Considering these facts, one must dismiss the feasibility of a determination of potency by an iodometric titration.

*Experimental.*—The reactions were performed in ground glass, stoppered bottles to prevent loss of iodine by vaporization. 0.01 N iodine and 0.005 N thiosulfate solutions were used. From 20 to 40 units of insulin, 5 cc. of phosphate buffer, 5 cc. of iodine solution, and 25 cc. of water were permitted to react under the conditions given in Table VII. A blank was always run simultaneously and the value of the iodine was taken from this figure. At a neutral or acid pH, the absorption varies with the time. At a pH of 6.8, 200 units of insulin absorbed 1.1 cc. of iodine solution in 5 minutes, 1.6 cc. in 40 minutes, and 2.0 cc. in 65 minutes. We found it very convenient to make a rapid titration in the following way. All the materials are added together in a pH 6.8 phosphate buffer. Sodium hydroxide is then added drop by drop until the iodine color disappears. After 5 minutes, acetic acid is added and the excess iodine determined. It will be noticed that the values obtained in this way are just as consistent as those obtained by the neutral incubation method.

(f) *Benzoylation.*—Scott (7) and Dingemans (11) both report experiments on the benzoylation of insulin. Using the Schotten-Baumann technique they obtain an ether-, acid-, and alkali-insoluble product with almost complete loss of potency. In a personal communication<sup>3</sup> Scott writes that in one instance he was able to reactivate the insulin through an acid hydrolysis. By using large amounts of benzoyl chloride, we have always obtained complete inactivation. When the amount of the benzoyl chloride was reduced, part of the potency was recovered

<sup>3</sup> "I have just looked up my laboratory notes on the benzoylation experiments. In these experiments I find that I had a precipitate settle out which was ether-, acid- and alkali-insoluble. In my published article I should have made this clear as the inference is that everything is acid-soluble. I did a great deal of work in trying to activate the different fractions after treatment with benzoyl chloride. In only one experiment out of a great number was I successful. In this experiment after removing the precipitate caused by the benzoylation I hydrolyzed the supernatant liquid with dilute hydrochloric acid in the usual way. I obtained a very active product which caused convulsions in rabbits. Numerous attempts to repeat this experiment were made, but with no success."

in the form of an acid-insoluble, alkali-soluble product. On standing a few days, the product lost its potency. Benzoyl chloride reacts with hydroxy, amino, and amido groups. It also dehydrates. Scott's reactivation experiment and the one in which we obtained an acid-insoluble, potent material appear promising. By modifying the conditions of experimentation, we hope to obtain larger yields of a more stable product.

*Experimental.*—1600 units of insulin, with a nitrogen content of 0.004 mg. per unit, were diluted to a volume of 40 cc. 1 gm. of sodium bicarbonate and 2 drops of benzoyl chloride were added and the solution shaken until the odor of benzoyl chloride had disappeared. The insoluble product was filtered off. It gave a faint biuret test and contained no potency. The filtrate was rendered acid and extracted with ether 3 times. The acid-insoluble, alkali-soluble, ether-insoluble residue had a potency of 300 units. On the following day the potency had dropped to 125 units and the 3rd day not more than 50 units were left. This product gave a strong biuret test.

1000 units of insulin were shaken, in a 50 cc. dilution, with 0.5 cc. of benzoyl chloride and 1 gm. of sodium carbonate for  $\frac{1}{2}$  hour. The mixture was made acid and extracted with ether to remove benzoic acid. The acid-insoluble residue in the aqueous layer was filtered off. It was alkali-insoluble and contained no potency. Attempts to hydrolyze this compound by heating in 90 per cent phenol at 70°C. were unsuccessful.

### *Sulfur Content of Insulin, and Other Proteins.*

From a study of the labile sulfur content of insulin preparations, Abel is inclined to believe that the "remarkably labile form" of sulfur is an essential constituent of the active hormone. This view is based upon the following evidence. The amount of more (0.1 N sodium carbonate) and less (10 per cent sodium hydroxide) labile sulfur found in insulin preparations is proportional to the unitage. Liberation of the sulfur by heating with phenol or with alkalis is accompanied by inactivation. Abel suggests that the only alternative interpretation of his findings is to assume that the insulin hormone is adsorbed upon a compound containing this labile sulfur. Abel's proposition, "Is insulin an unstable sulfur compound?" must be regarded as a contribution of the greatest importance because it was the first clue to any correlation between potency and chemical activity. It occurred to us that some light might be shed upon the subject by studying the effect of a whole series of chemical reagents upon

the labile sulfur. If the labile sulfur could be destroyed without affecting the potency, then the hormone would not be dependent upon the labile sulfur for its physiological activity. The converse of this proposition was not necessarily true, it being quite possible to destroy other chemical groups necessary for potency without affecting the labile sulfur group. Evidence indicating that the potency was destroyed whenever the sulfur was liberated would lend support to the theory that insulin is a labile sulfur compound. Abel's experiment of heating with phenol belongs in this category.

TABLE VIII.  
*Relationship Between Purity of Insulin and Labile Sulfur.*

Insulin.	Units per mg.	Nitrogen per unit.	0.1 N Na <sub>2</sub> CO <sub>3</sub> labile sulfur per unit.	0.1 N Na <sub>2</sub> CO <sub>3</sub> labile sulfur.	10 per cent NaOH labile sulfur.
		mg.	mg.	per cent	per cent
Brucine separation.....	55	0.0026	0.00051	2.8	3.6
I, purified.....	47	0.0030	0.00034	1.6	2.7
I.....	37	0.0038	0.00024	0.9	1.3
III.....	33	0.0043	0.00055	1.8	2.4
X, resin.....	33	0.0043	0.00055	1.8	2.7
Biuret-free.....	20	0.005	0.00175	3.5	6.4
IV.....	26	0.0055	0.00050	1.3	2.4
X, flocculent.....	26	0.0056	0.00049	1.3	2.3
Biuret-free.....	10	0.006			1.8
II, isoelectric.....	25	0.0057	0.00056	1.4	1.9
II, sac.....	21	0.0069	0.00066	1.4	1.7
Impure.....	3	0.045	0.02	0.65	1.4

We have been able to show that a large percentage of the sodium hydroxide labile sulfur, the amount representing the difference between the sodium carbonate and sodium hydroxide labile sulfur, is not involved in the potency. This was done by destroying the sulfur with very dilute nitrous acid without affecting the activity. We have never been able to destroy the sodium carbonate sulfur without destroying the potency. By using minimum amounts of iodine, we have destroyed the insulin without changing the sodium carbonate labile sulfur. Since an excess of iodine inactivates this labile sulfur, we have evidence



against the labile sulfur theory. There must be present in insulin a group more sensitive to iodine than the sodium carbonate labile sulfur. In Table VIII a comparison is made between the purity and the sodium carbonate labile sulfur content of twelve different insulin samples. It will be noted that in six of the twelve cases, the sulfur per unit is the same. This agrees with the finding of Abel. In the other five, excepting the very impure insulin, there is either considerably less or more sulfur per unit. The biuret-free insulin is very high in labile sulfur. If the labile sulfur is connected with the potency, limiting values should be obtained as the purity is increased. This is obviously not the case. It happens that under similar processes of purification, the unitage of the product may be proportional to the labile sulfur, or the iodine or peroxide titrations.

While our evidence does not support the labile sulfur theory, it contributes to our knowledge of the chemistry of the labile sulfur in the associated compounds. It has occurred to us that the labile sulfur may serve as a protective agent for the insulin. Both insulin and labile sulfur are very sensitive to chemical reagents. It is possible that, in the metabolism of the insulin, there arise conditions in which the labile sulfur, by reacting with a destructive agent, saves the insulin from inactivation.

*More and Less Labile Sulfur in Proteins.*—The term labile sulfur, which is found throughout the protein literature, is usually associated with the test by which it is detected; *viz.*, the formation of the black sulfide of lead when the substance is heated with alkali and a soluble lead salt. The labile sulfur may be regarded as that form of sulfur liberated from the protein as a sulfide by heating with alkalies. The detection as the sulfide of lead is a convenient way to inhibit the oxidation by air during the process of liberation. Abel and Geiling have found "that when one is dealing with a substance containing labile sulfur, one may be seriously mistaken in respect to its sulfur content if one makes the qualitative tests for sulfur in one way rather than in another." If the lead salt is added after boiling, all the sulfide may have been destroyed by oxidation and negative results will be obtained. Lead sulfide itself, in a finely divided state, is not immune to oxidation and may be destroyed under certain conditions of experimentation. The only safe way to make the test

is to use an atmosphere of inert gas. In our labile sulfur determinations we have eliminated the use of lead salt. The sulfide is liberated in an atmosphere of inert gas, and swept over into an oxidizing medium where it is determined as the stable sulfate. Piper, Allen, and Murlin (12) found that, "Heated with lead acetate and NaOH there is a slight browning of the fluid which perhaps indicates the presence of a trace of sulfur as an impurity." Yet we find that biuret-free insulin prepared according to Murlin's procedure contains 7 per cent labile sulfur. The discrepancy emphasizes the warning of Abel and Geiling quoted above.

Because we have been led to regard the labile sulfur in insulin as associated with the adsorbing proteins, a number of labile sulfur determinations has been made on several proteins under the identical conditions used for our insulin determinations.<sup>4</sup> There is much labile sulfur data in the literature but no two workers use the same conditions, so that the results are not comparable. The alkalinity used varies anywhere from 0.1 N carbonate to 30 per cent sodium hydroxide, and the time of heating from a few minutes to 9 hours. Johnson (13) gives an excellent summary of the labile sulfur literature in his article entitled "Sulfur linkages in proteins." We find that the labile sulfur in the insulin proteins is no more sensitive to alkalinity than the labile sulfur in keratin and zein. Gelatin and casein contain only a trace of sodium carbonate labile sulfur. The gelatin sulfur is remarkably stable even to 10 per cent sodium hydroxide. We were unable to determine the sodium carbonate labile sulfurs of lactalbumin and cottonseed globulin, because of the partial insolubility of our samples in this reagent. The sodium hydroxide sulfur of these substances is about half the total sulfur. Osborne (14) reports 13.0 per cent of the casein sulfur as loosely bound. This agrees with our sodium carbonate figure. We obtain a much higher percentage of labile sulfur in zein than is reported by Osborne. Our sodium hydroxide figure equals that for total sulfur. As has been stated, a direct comparison of results with other workers is not possible because of the difference in conditions of experimentation.

<sup>4</sup> We are indebted to the Protein Investigation Laboratory of the Bureau of Chemistry for a generous supply of purified proteins.

What do the more and less labile sulfur figures mean? Does the increased alkalinity break down a more stable sulfur or does it merely increase the speed of liberation of the same kind of sulfur linkage? In the case of cystine, we found that under our conditions of experimentation no cystine sulfur is liberated by 0.1 N sodium carbonate and only 2 per cent, or less than 10 per cent of the total sulfur, by sodium hydroxide. Longer heating set free increasing amounts of cystine sulfur. At least 90 per

TABLE IX.  
*Sulfur Content of Several Proteins and Related Compounds.*

Substance.	Sulfur.		
	0.1 N Na <sub>2</sub> CO <sub>3</sub> labile.	10 per cent NaOH labile.	Total.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cystine.....	Trace.	2.0	
Gelatin.....	"	0.005	0.7*
Casein.....	0.1	0.2	0.8
Lactalbumin.....		0.8	1.73*
Cottonseed globulin.....		0.3	0.62*
Keratin.....	2.2	2.7	5.0*
" azo.....	1.4	1.8	
Zein.....	0.4	0.7	0.6*
Insulin protein 3 units per mg.....	0.65	1.4	1.8
" purified 50 " " ".....	2.8	3.6	4.0
" biuret-free 20 units per mg.....	3.5	6.4	

\* Figures taken from literature.

cent of our labile sulfurs are therefore not due to cystine. If the remaining labile sulfur in proteins is of a single type, and the differences obtained with different concentrations of alkali are only a question of degree of reaction, then the ratios of the sulfurs obtained for different concentrations under otherwise identical conditions should be the same. A comparison of the results for keratin, zein, an insulin protein, and a purified insulin shows that this is not the case. By coupling an insulin and keratin with excess of diazo we were able to destroy all the insulin labile sulfur and only a fraction of the keratin labile sulfur. These results indicate that there are at least two other forms of labile sulfur in addition to the cystine form. See Table IX.

*Effects of Various Reagents on the Sulfur of Insulin.*—The effects of benzoyl chloride, acid and alkaline diazo compounds, phenylhydrazine, ultra-violet light, precipitation by heat, destruction by heat, trypsin, nitrous acid, and iodine upon the potency and sulfur were tried upon the same preparation of insulin. See

TABLE X.  
*Effect of Various Reagents on the Labile Sulfur of Insulin.*

Reagent.	Potency destroyed.	Sulfur.		
		0.1 N Na <sub>2</sub> CO <sub>3</sub> labile.	10 per cent NaOH labile.	Total.
	per cent	per cent	per cent	per cent
Insulin IV, 0.0055 mg. N per unit.....	0	1.3	2.4	3.8
“ benzoyl.....	100	0.5	0.8	
“ azo.....	100	0.2	0.3	
“ “ acid.....	25	1.1	1.6	
“ phenylhydrazine.....	60	1.3	1.6	
“ ultra-violet, H, 40 hrs.....	75	1.0	1.6	
“ “ “ 80 “ .....		0.5		
“ “ “ N, 17 “ .....	48	0.8	1.2	
“ “ “ 32 “ .....	65	0.9	1.4	
“ “ “ 48 “ .....	75	1.2	1.6	
“ heat-precipitated.....	0	1.6	2.6	
“ heat-destroyed.....	100	1.3	1.4	
“ trypsin.....	100	0.5	1.2	
“ “ .....	100	0.6	1.2	
“ “ acidified.....	100	0.7		
“ nitrite.....	15	0.9	1.2	
“ “ .....	30	1.1	1.5	
“ iodine.....	80	1.1	1.3	
“ I, 0.0030 mg. N per unit.....	0	1.6	2.7	
“ nitrite.....	0	1.4	1.9	
“ “ .....	50	1.4	1.8	

Table X. This insulin was made by our usual method of purification and analyzed 0.0055 mg. of nitrogen per unit. In every case in which the insulin was inactivated, about half the sodium hydroxide labile sulfur was destroyed. The agents which destroyed the potency were benzoyl chloride, alkaline diazo compounds, phenylhydrazine, ultra-violet light, heat, trypsin, and

iodine. In addition, benzylation, coupling, ultra-violet light, and trypsin affect the carbonate sulfur. When the insulin is destroyed by heating in acid solution, there is no change in the sodium carbonate sulfur. Abel and Geiling made the same observation upon their insulin. They do not report on the sodium hydroxide sulfur. Before we had succeeded in destroying the sodium hydroxide sulfur without affecting the potency, we were inclined to believe that this form of sulfur was involved in the active insulin group. The observation that anything which destroyed the potency also destroyed this form of sulfur appeared very significant. We almost overlooked the fact that in another insulin (see Table VIII, Insulin II) there is only a very small difference between the sodium hydroxide and sodium carbonate sulfurs.

When the insulin was inactivated by exposure to ultra-violet light, there was at first a marked decrease in the sodium hydroxide and sodium carbonate labile sulfurs, followed by a small but definite increase of both these values. At the end of 17 hours, half the potency was destroyed with an absolute 0.5 per cent decrease in sodium carbonate labile sulfur. 15 more hours of exposure destroyed 15 per cent additional potency, and 30 more hours, an added 25 per cent of the potency. The sodium carbonate labile sulfur at the end of this period was raised almost to its initial value. This is evidence for the occurrence of at least two reactions in the insulin complex. The experiment was performed in an atmosphere of nitrogen. An autooxidation-reduction is brought about by the action of the ultra-violet light, the active insulin group being reduced at the expense of the labile sulfur. When the potency has been destroyed, the labile sulfur is reduced at the expense of some other part of the molecule. This is an explanation based upon the facts; but there is no absolute proof for it. Ultra-violet light will attack almost any organic compound, even the most stable of chromophoric groups. What happens is usually a matter of speculation. Ultra-violet light changed neither the pH of the insulin nor the test for cystine.

The experiments relating to the action of trypsin upon the labile sulfur were significant because they showed that the sodium carbonate labile sulfur was already liberated by the trypsin, and that acidification, without the carbonate heating, gave the same

amount of sulfur as heating with carbonate in the usual way. Some sodium carbonate labile sulfur and a large percentage of sodium hydroxide labile sulfur are destroyed by tryptic digestion, possibly through oxidation by the air. Johnson (15) has suggested that the labile sulfur may be present in protein as a thio-carbonyl linkage in thiopolypeptides and dithiopiperazine derivatives. It is interesting to conceive of trypsin attacking these compounds and disrupting them through the elimination of hydrogen sulfide.

Phenylhydrazine is cited by Laqueur (16) as an insulin precipitant. We found that it destroys about 60 per cent of the potency and a large percentage of the sodium hydroxide labile sulfur; it also renders the product acid-insoluble. Attempts at regenerating the insulin by separating the phenylhydrazine through acetone, as accomplished in sugar chemistry, were unsuccessful.

The great importance attached to the results with nitrous acid led us to repeat the experiments upon another sample of insulin. We used Insulin I, purified, which had twice the potency, per nitrogen basis, of the other preparation. The results were just as significant. When small amounts of nitrous acid were employed, none of the potency was destroyed. The sodium hydroxide sulfur was lowered from 2.7 to 1.9 per cent and the carbonate sulfur was practically unchanged. When more nitrous acid was used, 50 per cent of the potency was destroyed but there was no further change in the sulfur values. This is another instance of inactivation of the active group through some group other than the labile sulfur.

*Experimental.*—The micro method devised in this laboratory for the determination of the labile sulfur is described in a separate paper (17). We have followed Abel's conditions of using 0.1 N sodium carbonate and 10 per cent sodium hydroxide in an atmosphere of nitrogen. In Abel's method, the sulfur is separated by contact with plumbite. Preliminary experiments showed that the separation as lead sulfide was unnecessary. We check Abel's cystine value exactly. At least seven of our insulins, which were of the same degree of purity as Abel's, yielded figures very similar to those obtained by him, the sodium carbonate sulfurs approximating 1 per cent, the sodium hydroxide 2 per cent, and the total sulfur 3 per cent. With the exception of the biuret-free insulin, all of the data pre-

sented have been checked from 2 to 5 times. The preparations of the chemical insulin derivatives used in the labile sulfur analyses have been considered under their respective headings. The keratin azo compound was prepared under the same experimental conditions as the insulin derivative. In all the samples used for sulfur analyses, special care was exercised in separating excess reagent from the sample. The samples treated with nitrous acid were not analyzed for sulfur until they gave a negative test with starch-potassium iodide solution. The benzoylated product was extracted with ether and centrifuged from dilute alkaline solution. It might be argued that the sulfur analyses are lower in the chemically treated insulins, not because the sulfur has been destroyed, but because the sulfur liberated in the alkaline heating reacts with the new chemical derivative. In answering this objection it is necessary to consider each case separately. Since the carbonate sulfur before and after nitrous acid treatment is the same, the possibility of a reduction of a nitroso compound is negatived. The possibility that the sulfide reacts with the azo group is remote, it being a common practice to reduce nitroazo compounds to aminoazo compounds with sulfide. For this reason the use of para-nitrobenzenediazonium chloride and the arsenic analogue of sulfanilic acid, though excellent coupling reagents for insulin, was avoided. The experiments with ultra-violet light were performed in a quartz tube in an atmosphere of nitrogen.

### *General Considerations.*

Our usual preparations of insulin respond to the customary protein reactions and are precipitated by the ordinary protein precipitants. This purified insulin is soluble on either side of the isoelectric point, which lies at a pH of 5.8 to 6.0 (colorimetric). Most of the active material is not dialyzable. Sulfur is present in other forms beside cystine. Phosphorus is absent. Carbohydrate is not present. Pepsin and trypsin inactivate the insulin. Hydrolysis with acids yields a distribution of the amino acids which is characteristic of protein and of insulin, as reported by others.

Certain observations suggest that insulin is not a protein but that the properties just mentioned are chiefly due to contaminating protein material. The nitrogen content of several preparations is low for a typical protein. Values ranging from 12 to 13 per cent are not uncommon. Higher values were found for tyrosine and cystine than others have reported. A sample analyzing 25 units per mg. showed 17.9 per cent tyrosine and 7.1 per cent cystine by the method of Folin and Looney (18). A different preparation, which also contained 25 units per mg.,

gave values of 12.9 per cent tyrosine and 6.6 per cent cystine. These may be compared with Scott's figures of 10.0 per cent tyrosine and 0.7 per cent cystine. The higher values indicate a concentration of these amino acids in the particular insulins examined. In these preparations one-fifth of the material was composed of the two amino acids. Such values are certainly unusual for proteins. Several experiments already presented have shown that very small amounts of different reagents inactivate the insulin. The quantities required to inactivate insulin are much smaller than the amounts necessary to react completely with the total complex. Such experiments indicate that the physiologically active group comprises but a fraction of the ordinary purified preparations of insulin.

Murlin (6, 19) has reported the preparation of biuret-free insulin from pancreas perfusates. The earlier observation was severely criticized by various workers who maintained that not enough material was used to obtain the biuret test. In his second paper, Murlin has met this objection by using such large amounts of material as to leave no doubt about the correctness of the observation. We have prepared biuret-free insulin from perfusates according to Murlin's method and from our purified insulins by application of the same principles. 20 units of ordinary protein insulin having a potency of 50 units per mg. give a positive biuret test. 80 units of our biuret-free insulin do not give the faintest trace of a test under the same conditions. Whether this biuret-free insulin is the partially inactivated hormone, freed from protein, or whether it is merely a denatured protein, has not been established. But the fact remains that the physiologically active group is not dependent for its activity upon the characteristic biuret linkages.

#### SUMMARY.

A method for the preparation of insulin is described. This method gives a good product with comparatively less effort than is required by other procedures.

Various methods of purifying crude insulin are discussed. Fractional precipitation with alcohol is a very efficient treatment



for eliminating inactive material and for concentrating the hormone.

The action of several chemical reagents upon insulin is considered. The important fact established by these experiments is that the quantities of several different reagents required to inactivate insulin are much smaller than the amounts necessary to react completely with the total complex. The experiments indicate that the physiologically active group comprises but a fraction of the ordinary purified preparations of insulin.

A rather complete study of the labile sulfur of insulin and other proteins was made. The results of these experiments indicate that the apparent relationship between the activity of insulin and the labile sulfur is fortuitous. A large percentage of the sodium hydroxide labile sulfur can be destroyed without affecting the physiological activity. Furthermore, the potency of insulin can be destroyed without changing the sodium carbonate labile sulfur.

The hormone, insulin, probably comprises but a fraction of all purified preparations which contain protein.

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## STUDIES ON THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.

### V. THE TEMPERATURE CORRECTION IN CATALASE DETERMINATIONS.

BY SERGIUS MORGULIS AND M. BEBER.

*(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)*

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In a previous paper (1) it was shown that the activity of a catalase preparation varies greatly with the temperature at which the reaction is carried out, and we were led to conclude that the optimum temperature lay somewhere between 0 and 10°C. Our experiments demonstrated definitely that the maximum catalase activity does not occur at 0°C., as is assumed by some investigators. At that time the thermostat we used in our work was not efficient either in maintaining an unchanged temperature, especially over a long period of time, or in regulating the temperature at any desired level. We were, therefore, unable then to determine the *exact* temperature at which catalase displayed its optimum activity. To establish this point definitely we constructed a thermostat, shown in Fig. 1, where, by combining mechanical devices for simultaneous cooling and heating of the water, we could maintain any desired temperature with a maximum variation of 0.1°C. and for any length of time. The thermostat is a strong wooden box lined with thick sheet lead in which the water is constantly cooled by means of a Kelvinator freezing machine<sup>1</sup> (the copper expansion coil which runs along the inside walls of the box is not clearly seen in the photograph). The desired experimental temperature is secured by means of two carbon filament lamps (300 watts) placed on the bottom, which serve as heating units. These are operated through a relay which is

<sup>1</sup> The Kelvinator freezing machine has been kindly loaned to us by the Nebraska Power Company.

controlled by a thermoregulator. The latter consists of a large oblong bulb, containing  $\text{CCl}_4$ , attached to a capillary tube filled with mercury and bearing two platinum contact wires about 2 inches apart. The thermoregulator is removable and can be easily set for any temperature by varying the amount of mercury in the capillary, and, opening or closing the circuit automatically as the temperature of the water rises above or falls below that for which it is adjusted, maintains a constant temperature within

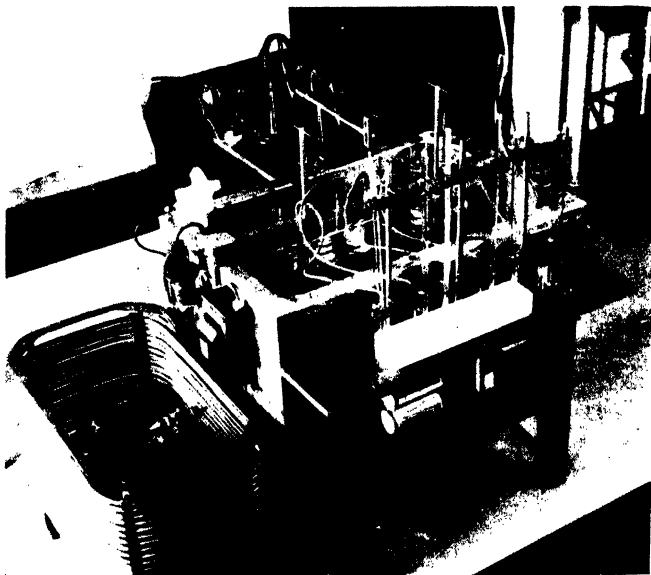


FIG. 1.

the thermostat. The water in the thermostat is constantly stirred by a continuous stream of air.

The experimental procedure in the present study is essentially the same as has been used in the earlier determinations. The tests were made with a semipure beef kidney catalase preparation which had retained its full strength for 3 years. The pH was invariably 7.0 and was maintained with the Kolthoff phosphate-borax buffer; the hydrogen peroxide was used in 0.50 to 0.51 N concentration, and the total volume of the mixture was 20 cc. The bottles in which the reaction took place were completely

submerged in the thermostat and shaken rhythmically by the rotating device shown in Fig. 1.

We performed altogether about 225 experiments at temperatures ranging from 0–30°C. As the temperature increased it was necessary, of course, to increase also the catalase concentration since more was destroyed during the experiment. In Fig. 2 a number of experimental results are recorded graphically showing the relation between catalase activity and temperature

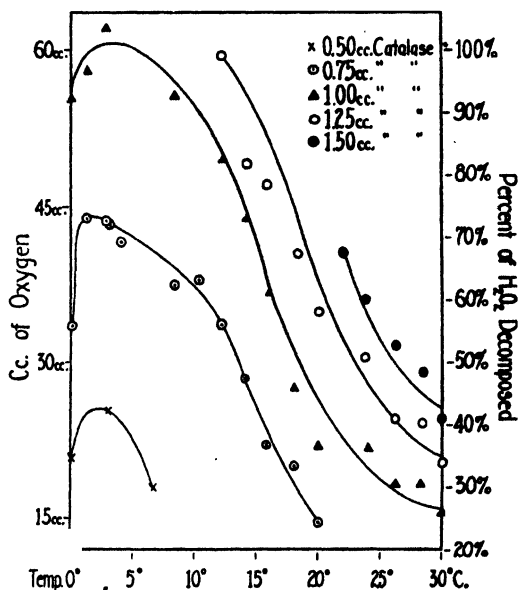


FIG. 2.

with varying amounts of our catalase preparation (diluted 1 to 100). The results, obtained at temperatures 2° apart, which were maintained constant within 0.1°C. over the entire experimental period, corroborate our earlier observations, not only as to the low activity at 0°C. and the lowering of activity with the rise in temperature, but also establish definitely the optimum temperature at 1–3°C. The variation within this temperature range is so slight that we may set the optimum temperature at the mean, 2°C. The decrease in catalase activity found at 0°C.

is being studied at present and we hope to be able to elucidate this question in the future.

In Fig. 3 we have arranged the experimental findings at temperatures from 2–30°C. in such a way that the per cent of hydrogen peroxide decomposed is plotted against the relative catalase concentrations. When these experimentally determined points are connected, we get a series of straight line curves, each corresponding to a different temperature. These curves are, therefore, catalase isotherms. Two interesting points emerge from Fig. 3: first, the increasing tendency of the isotherm curves to flare out as we pass from the lower to the higher tem-

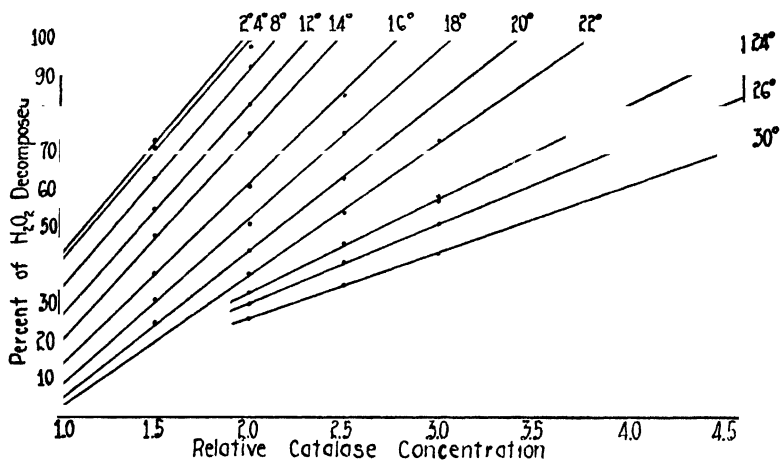


FIG. 3.

peratures; secondly, the distance between successive isotherms for equal temperature intervals is not the same at different temperature ranges. The isotherms for 1–3° coincide so completely that we plot only the values for the mean temperature (2°C.). The other isotherms group themselves into three distinct zones: first zone, from 2–12°C., with a gradually increasing separation of the isotherms; a second zone, from 12–22°C., with practically equal horizontal displacement of the curves for similar temperature intervals; and, a third zone, from 22–30°C., where the horizontal displacement abruptly changes to more than twice that in the previous zone.

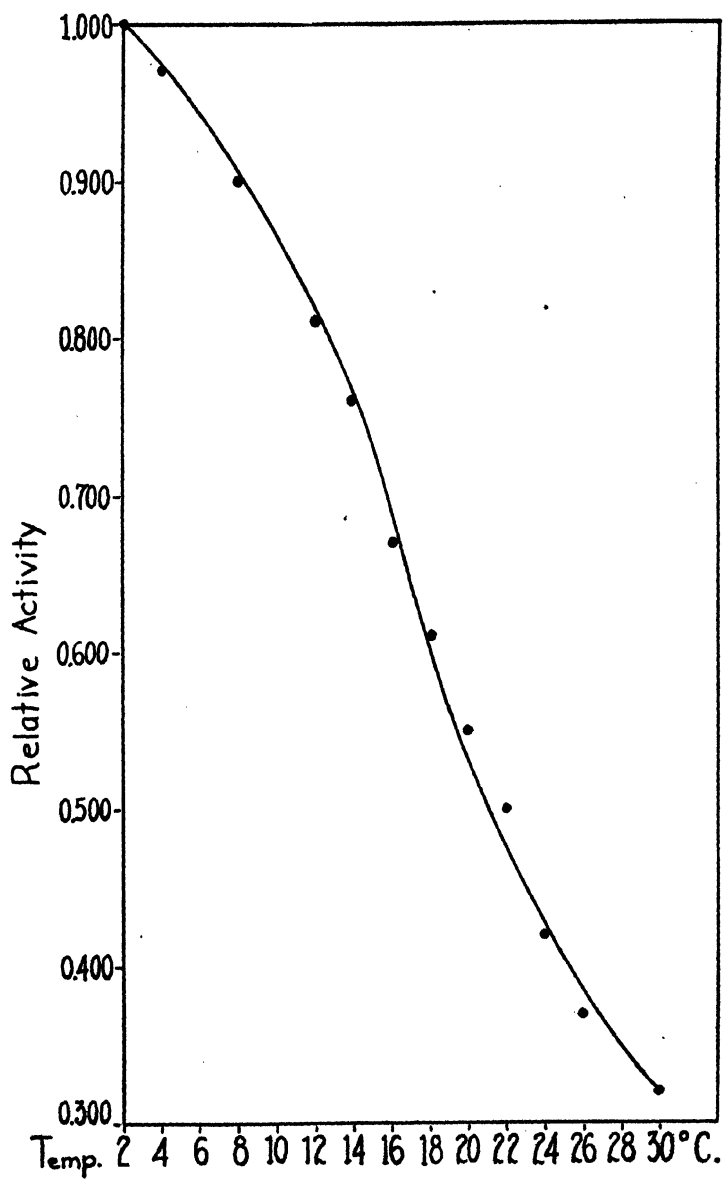


FIG. 4.



Drawing the horizontal line corresponding to 70 per cent decomposition of the hydrogen peroxide, we can read on the abscissa the relative catalase concentration which will manifest this activity at various experimental temperatures. The activity represented by 70 per cent decomposition of peroxide is selected, because, as was shown by one of us (2), the course of the catalase reaction at this point follows exactly that of a bimolecular isotherm, and had already previously been proposed as a means for standardizing catalase preparations (3). This method of standardization gives absolutely reproducible and predictable results. It is obvious from Fig. 3 that to produce the same amount of work (*i.e.* decompose a definite quantity of hydrogen peroxide) four times as much enzyme is required at 30° as at 4°C., etc. Assuming that all of the catalase is active at the optimum temperature, it is easy to calculate how much of the catalase undergoes destruction at higher temperatures; or, regarding the amount of catalase which at 2°C. exerts 70 per cent decomposition as an enzyme unit, we thus obtain the relative amount which is still active at any other temperature. Plotting the values for the relative catalase activity thus calculated against temperature, we get a curve, Fig. 4, of remarkable regularity which corresponds to the equation<sup>2</sup>  $y = 0.58 + 0.425 \sin \frac{\pi}{10} (19 - t)^\circ$  where  $y$  is the relative activity, and  $t$  the experimental temperature. The points calculated from this formula coincide perfectly with the experimental points down to a temperature of 24°C. From this point, however, the calculated curve declines much more abruptly than does the experimental curve. This is of little practical significance since the catalase reaction is not generally studied at such high temperatures.

The deviation of the experimental from the calculated curve occurring at 24°C. has a theoretical interest worth pointing out here. In an earlier paper (4) it was shown that the catalase destruction is a monomolecular reaction with a temperature coefficient which increases moderately for higher temperature ranges. The temperature coefficient for the catalytic reaction, on the contrary, has no fixed value but increases greatly as the

<sup>2</sup> It is a pleasure to acknowledge our indebtedness to Dr. M. G. Gaba, Professor of Mathematics in the University of Nebraska, for working out the equation.

experimental temperature rises (1). It follows from our new observations that 24°C. is a critical temperature for the catalase reaction, the rate of the catalytic reaction ( $E_A + H_2O_2 \rightarrow E_R + O_2$ ) being so much greater than the enzyme destruction reaction ( $E_A + H_2O_2 \rightarrow E_O + H_2O$ ) that the volume of oxygen set free becomes progressively greater than the theoretically anticipated amount for temperatures above 24°C.

This curve, Fig. 4, makes it possible now to find the actual number of enzyme units no matter at what temperature the experiment is actually performed. It is not practicable to conduct catalase experiments at temperatures ranging from 1–3°C. except where a special thermostat is available, and even then it is not desirable to run experiments at such low temperatures because they are very time-consuming, many hours being required for the completion of the reaction. A range from 16–20° is probably most suitable for laboratories with ordinary equipment, and the reaction, besides, is completed within 30 to 90 minutes. The results obtained at any of these temperatures can be easily corrected for the catalase destroyed at the temperature above the optimum. It is necessary, of course, to determine the per cent of hydrogen peroxide decomposition by at least two different catalase concentrations. This should not exceed 90 per cent, but should preferably fall within the range of 50 to 80 per cent of the total peroxide used. With these two points available the catalase isotherm may be established (see Fig. 3) and the catalase concentration producing an exact 70 per cent decomposition determined on the abscissa at the point of intersection with the corresponding ordinate. The relative activity at the experimental temperature is read directly from the curve, Fig. 4. Thus, at 21°C. 0.50 of the catalase used is active; at 14.5°C., 0.75; and at 8°C., 0.90, etc. The quantity of catalase producing 70 per cent decomposition ( $Q$ ) divided by the factor for relative activity ( $A$ ) at the temperature of the experiment gives the actual amount of catalase used in the test. Or, assuming  $Q:A$  at 2°C., where  $A=1$ , as the catalase unit, we determine the number of enzyme units. Any catalase preparation can thus be standardized on a strictly quantitative basis which is easily and exactly reproducible.

To check our conclusions, we calculated the amount of our catalase preparation which would be necessary to yield 40 to 90 per cent decomposition of hydrogen peroxide at any temperature (under standard experimental conditions). The results of these experiments with the estimated quantities brought perfect verification of the predicted values.

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# THE EFFECT OF ACID AND BASE INGESTION UPON THE ACID-BASE BALANCE.\*

BY ALFRED E. KOEHLER.

(From the Medical Clinic of the Massachusetts General Hospital, Boston.)

(Received for publication, November 12, 1926.)

Previous to the conception of hydrogen ion concentration as an index of acidity or alkalinity, only hazy notions existed as to the reaction of the body fluids. Soon after the acid-base balance was more fully comprehended, it became a prevalent idea that the reaction of the blood and tissues was maintained within exceedingly close limits and that any appreciable variation would be incompatible with life. Haldane (1) in 1922, speaking of the pH of the blood, wrote:

"Hasselbalch estimates that a difference of .03 can be detected in single determinations by the electrometric method; but this is a very large difference, corresponding to an increase of 250 per cent in the breathing. Time and effort will continue to be wasted on futile measurements until the extreme fineness of the physiological regulation of pH in the blood and tissues is more fully realized."

He further states: "A continued difference of 0.1 in pH would in all probability cause danger to life. This is a much lower limit than has commonly been assumed."

These conclusions were based on many careful observations but direct pH determinations were few. For example, Davies, Haldane, and Kennaway (2) studied the effect of the ingestion of 500 cc. of 0.116 N HCl taken within 80 minutes but noticed no fall in the alveolar CO<sub>2</sub> tension. Similarly, 30 gm. of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O caused no fall. However, Hasselbalch (3) as early as 1912 demonstrated that a meat diet increased the relative acidity of the blood over that of a vegetable diet by as much as 0.02 pH.

\* This paper is one of a series of studies on metabolism from the Harvard Medical School and allied hospitals, a part of the expense of which has been paid by the Proctor Fund for the study of chronic diseases.

More recently Haldane (4) conducted an experiment where  $\text{NH}_4\text{Cl}$  was ingested, 20 gm. the first 2 days and 15 gm. the 3rd. The  $\text{CO}_2$  capacity of the blood at 40 mm. Hg dropped from 48.1 before, to 28.2 volumes per cent at the end of the 3rd day, with a similar drop in the alveolar  $\text{CO}_2$  tension from 38.8 to 27.8 mm. Hg. He figured that 96 per cent of the acidosis was compensated by increased respiration. Similarly, Haldane, Hill, and Luck (5) showed that 85 gm. of  $\text{CaCl}_2$  taken over a period of 3 days caused a markedly acid urine with increased  $\text{NH}_3$  output and a drop in the alveolar  $\text{CO}_2$  tension.

It was my fortune to have under observation a group of patients suffering from chronic lead poisoning, who were being treated by Dr. J. C. Aub for long periods with large doses of acids or alkalis, or acid- or alkali-producing substances for the purpose of increasing the elimination of lead. Aub and his coworkers (6) have shown that lead is deposited in the skeleton largely as a calcium lead phosphate and that its minimal solubility is approximately at the normal pH of the blood with increasing solubility in either direction of the pH range. During the medication of these patients the acid-base balance of the blood was carefully studied.

### *Methods.*

The patients in all of these experiments were on a rest schedule which consisted of lying in bed or sitting in a chair. Occasionally short walks up or down the ward or hall were permitted. Ascending and descending a stairway leading from the ward was used as an exercise test.

The diets were constant throughout the experiments and control periods and consisted of protein, 1.25 gm. per kilo, carbohydrate 1200 to 1800 calories, and fat 300 to 600 calories in 24 hours. The diet consisted largely of potatoes, bread, butter, sugar, meat, and fruit. The last consisted of 100 gm. each of apples and bananas per day. The Ca intake was low, approximately 100 mg. per day unless separately added to the diet as indicated under medication. Fluid intake was limited to approximately 3500 to 4000 cc.

The various acids, alkalis, or salts were given in water in rather dilute solutions from 8.00 a.m. to 6.00 p.m. distributed over 2 hour intervals.

Unless otherwise specified, the blood samples were taken at about 5.00 p.m. Venous blood and in one case arterial was taken from the arm directly under paraffin oil over powdered oxalate. The blood was chilled immediately in ice water and the determinations were done within 30 minutes. The pH and  $\text{CO}_2$  determinations were done directly on the whole blood, as described in previous papers by the writer (7). The  $\text{CO}_2$  content was determined by the Van Slyke constant volume apparatus and the pH by means of the hydrogen electrode at  $37.50^\circ\text{C}$ . The hydrogen which was equilibrated with the blood contained 5.5 per cent  $\text{CO}_2$ . The  $\text{CO}_2$  tension was calculated from the Henderson-Hasselbalch equation using 0.0587 as the solubility factor for  $\text{CO}_2$  in blood and a pK value for whole blood of 6.15.

The respiratory volume per minute was determined by means of a rebreathing soda-lime spirometer of the Benedict-Collins recording type with chronograph attached.

#### EXPERIMENTAL.

##### *Administration of Acids or Acid-Producing Substances.*

The first series of observations was made upon P. M-n during a period of  $\text{H}_3\text{PO}_4$  administration. This patient was a male, 48 years of age, Greek, and had been a rubber mixer for the 7 preceding years. The acute symptoms of severe abdominal colic and marked ease of fatigue had subsided but a bilateral wrist drop and a general muscular weakness were still present. At the time of observation the patient had been hospitalized for 2 months and the hemoglobin and red blood cell count had increased from 75 to 85 per cent and 3,700,000 to 4,500,000 respectively. During this time the non-protein nitrogen had decreased from 51 to 36 mg. per 100 cc. of blood and the phenolsulfonephthalein excretion averaged well over 50 per cent for 2 hour periods.

The variations in venous blood pH, total  $\text{CO}_2$  and  $\text{pCO}_2$  together with volume respiration are also shown graphically in Fig. 1. The amount of  $\text{H}_3\text{PO}_4$  that could be tolerated by mouth was approximately 17 gm. per day so from the 11th day of medication on, an additional amount of  $(\text{NH}_4)_2\text{HPO}_4$  was given to see whether this would act similarly to  $\text{NH}_4\text{Cl}$  and produce a further drop in pH. However, such an action was not noticed to any

marked degree with the addition of 4 to 8 gm. of  $(\text{NH}_4)_2\text{HPO}_4$ . From approximately the 14th day to the 25th day, the acidosis remained at about a level with a pH of approximately 7.20 and a total  $\text{CO}_2$  content of 28 volumes per cent. The minute volume respiration increased from 6 before acid ingestion to approximately 10 liters during the acidosis, while the venous  $\text{pCO}_2$  dropped from about 44 to 38 mm. Hg. On the 22nd day an arterial blood sample was obtained and the pH was 0.02 higher and the total  $\text{CO}_2$  and  $\text{pCO}_2$  less in about the normal ratio. Weakness, headaches, and loss of appetite were noted during the course of the medication but

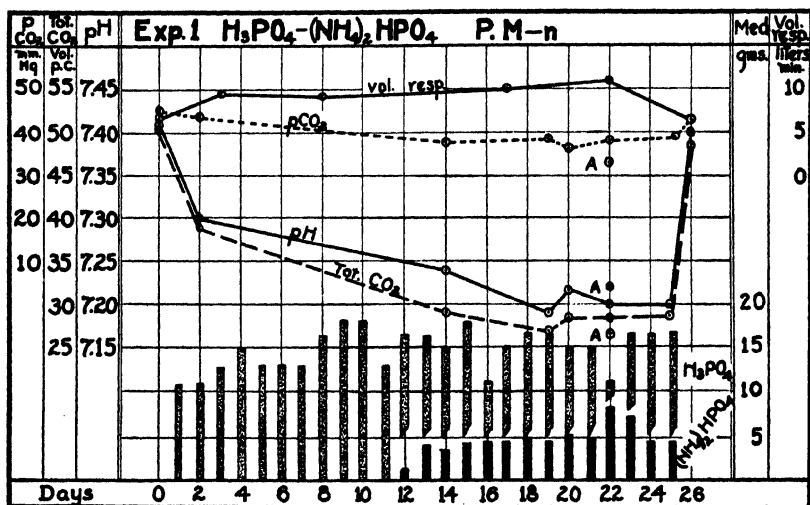


FIG. 1.

in general the condition of the patient was quite good while at rest. When any exertion was attempted, however, marked distress resulted. The recovery from the acidosis was rapid and complete.

Fig. 2 shows the results of  $\text{H}_3\text{PO}_4$  medication upon this same patient 2 months later. The results were very similar except that as much acid was not given and the pH and total  $\text{CO}_2$  did not drop as far. From the 10th day on, calcium lactate was also given orally but not simultaneously with the acid. This was done to ascertain the effect of a positive Ca balance upon the lead excre-

tion. The effect of the calcium lactate was nearly a complete neutralization of the acidosis, undoubtedly due to the formation

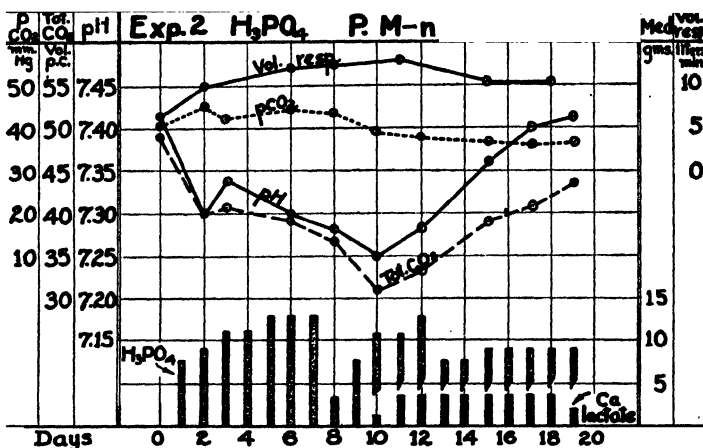


FIG. 2.

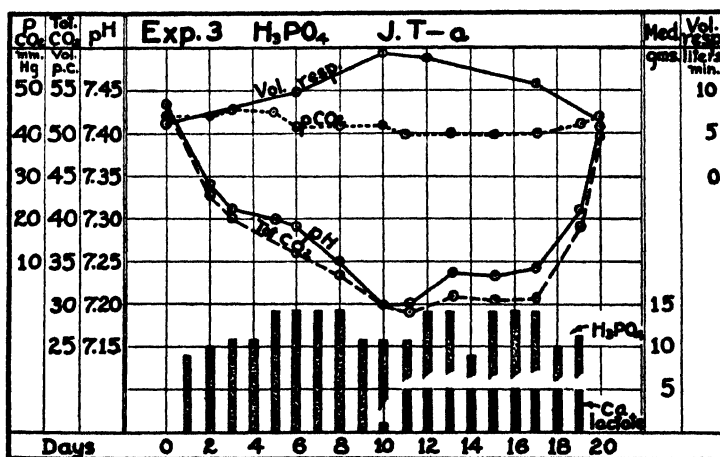


FIG. 3.

and excretion of calcium phosphate. During this period of acidosis as well as the previous one, there was a definite loss in weight



of about 8 per cent of the total. The greater part of this occurred the first 4 or 5 days and was undoubtedly due to dehydration as there usually was a definite polyuria and purgation during this time but not later.

A similar phosphoric acid acidosis was obtained in the case of J. T-a. This patient was a male, 34 years of age, Lithuanian and had been a rubber mixer for the 5 preceding years. The acute symptoms of lead poisoning had subsided a month before this period of acid medication but a bilateral wrist drop of moderate grade with some weakness of the muscles of the upper arms and

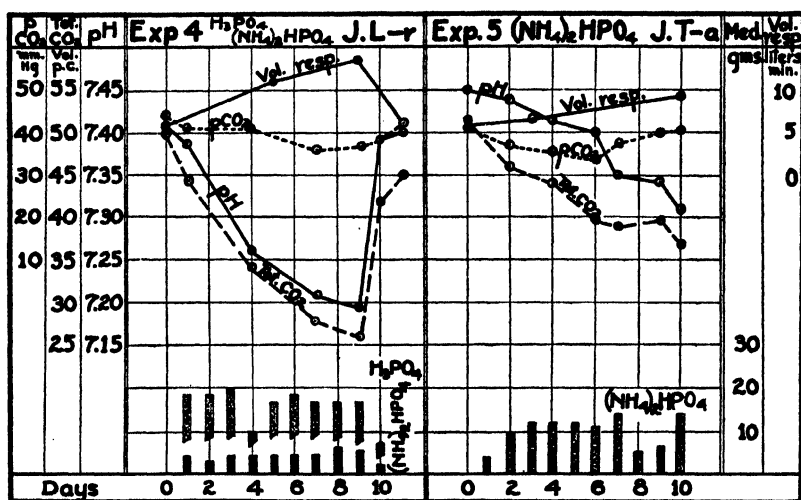


FIG. 4.

shoulder girdle remained. The hemoglobin was 75 per cent and the red cell count 3,900,000 with marked stippling of cells. The renal function and blood non-protein nitrogen were normal. The results of this experiment are shown in Fig. 3. The findings were very similar to those obtained in the case of P. M-n. Inasmuch as the blood samples were usually taken toward the end of the day of medication and no acid was given during the night, in this case on the 11th day a blood sample was obtained in the morning before any acid was given. These results, compared with those obtained after about four-fifths of the acid was given, show that there is quite a marked diminution of the acidosis overnight.

Experiment 4, Fig. 4, shows the results of  $\text{H}_3\text{PO}_4$  and  $(\text{NH}_4)_2\text{HPO}_4$  ingestion for a period of 10 days in the case of J. L-r. This patient was a male, 32 years of age, Austrian, and his occupation was that of a chauffeur. His complaint had been severe generalized abdominal cramps. Examinations showed a pale individual with a marked alveolar lead line. There was no muscle weakness. The red blood cell count was 3,600,000 and hemoglobin 65 per cent at time of admission but there was an increase up to 4,000,000 and 75 per cent respectively 3 weeks later at the time of study. There was no evidence of any renal insufficiency. An average of approximately 15 gm. of  $\text{H}_3\text{PO}_4$  and 4 to 7 gm. of

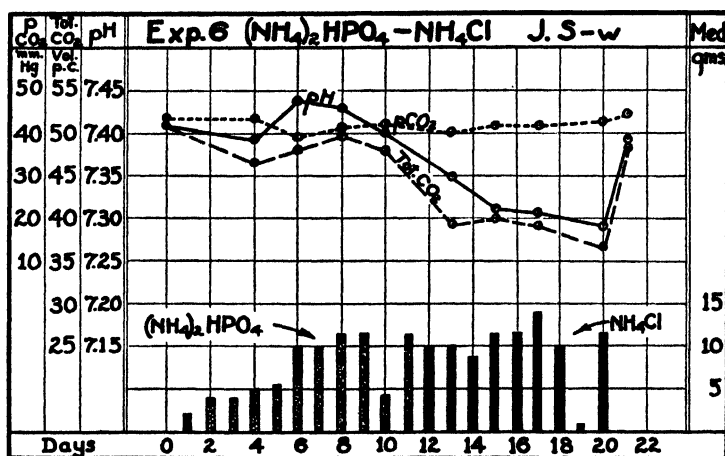


FIG. 5.

$(\text{NH}_4)_2\text{HPO}_4$  daily for 9 days produced an acidosis below pH 7.20. At the end of this period the patient noticed considerable lassitude, headaches, muscle pains, and marked respiratory distress upon exertion. There was practically a complete return to normal 24 hours after the acid was stopped.

Experiment 5, Fig. 4, shows in the case of J. T-a (2 weeks after the experiment shown in Fig. 3) the effect of daily doses of 6 to 14 gm. of  $(\text{NH}_4)_2\text{HPO}_4$ . Only a very moderate acidosis resulted and there was no particular symptomatic upset beyond slight purgation the first few days and moderate lassitude toward the close of the 10 day period.

Fig. 5 also shows the results of  $(\text{NH}_4)_2\text{HPO}_4$  ingestion in the case of J. S-w. This patient was a Negro, male, 30 years of age. He recently had mild abdominal colic and nausea. Examination revealed a well built, strong individual with a definite alveolar lead line. This patient had had a posterior gastroenterostomy a year previously for a bleeding gastric ulcer. At the time of the present admission all evidence suggested that the ulcer was healed. The hemoglobin was 78 per cent and the blood red cell count 3,900,000. There was no evidence that showed any renal insufficiency. During the first 11 days of  $(\text{NH}_4)_2\text{HPO}_4$  ingestion

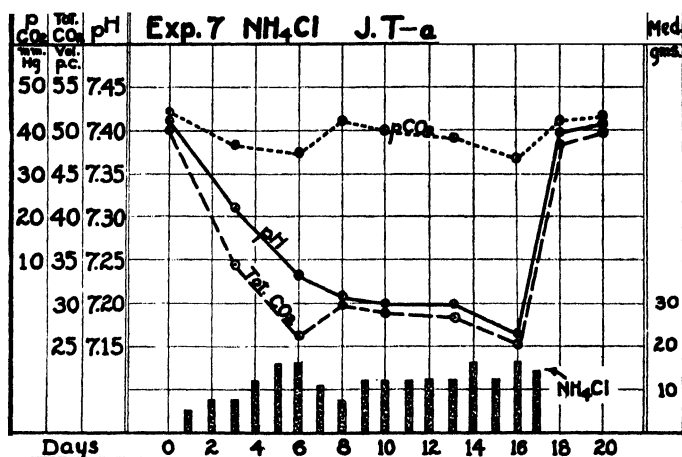


FIG. 6.

there was only a slight shift in the reaction of the blood in the direction of an acidosis. During the 6th and 8th days there was a slight shift toward the alkaline side, the cause of which remained unexplained. During this period there was no symptomatic change. On the 12th day of the experiment, 10 gm. of  $\text{NH}_4\text{Cl}$  were substituted for the phosphate. Very shortly the total  $\text{CO}_2$  dropped together with the pH and the respiratory minute volume increased to 10 liters per minute. Lassitude, headaches, and loss of appetite developed and the acid feeding was discontinued on the 20th day with a quick return to normal.

The effect of prolonged administration of  $\text{NH}_4\text{Cl}$  is shown in

Experiment 7, Fig. 6. An average of approximately 12 gm. was given daily for 17 days. There was a rapid drop in the blood pH and total  $\text{CO}_2$  for the first 6 days and then there was a gradual decrease until the end of the 16th day. In this case an acidosis in the vicinity of pH 7.20 was maintained for about 9 days with a minimal value of pH 7.17. During the period of  $\text{NH}_4\text{Cl}$  feeding the respiratory volume increased from 7.80 to 15.05 liters per minute. This patient noticed considerable lassitude and moderate headaches during the last 12 days of the experiment. He desired to remain in bed continuously and at times appeared quite list-

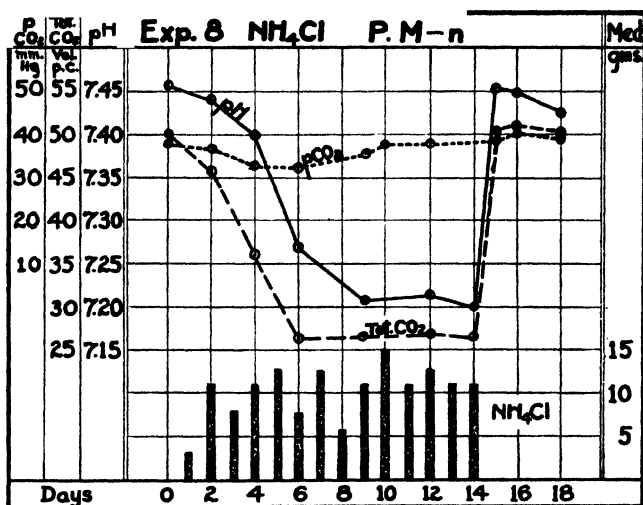


FIG. 7.

less. During the last few days he complained of considerable epigastric distress with occasional abdominal cramps. The recovery following this period of acidosis was not quite as rapid as usual, for even 48 hours after the  $\text{NH}_4\text{Cl}$  was stopped, moderate lassitude and weakness persisted.

Experiment 8, Fig. 7, shows a similar response to  $\text{NH}_4\text{Cl}$  ingestion in the case of P.M.-n. In this case the daily amount of  $\text{NH}_4\text{Cl}$  averaged about 8 gm. per day for 14 days. Diuresis and thirst were fairly marked during the first few days of the experiment but disappeared later. The recovery, particularly as to lassitude and

weakness, was not quite complete at the end of the 2nd day but a normal state had practically been reached by the end of the 3rd day.

The effect of  $\text{NH}_4\text{Cl}$  upon P. M-n as recorded in the last experiment was again observed 2 weeks later under similar conditions and is shown in Experiment 9, Fig. 8. This time the patient tolerated only about 10 gm. of  $\text{NH}_4\text{Cl}$  daily and even then he developed headache, backache, and loss of appetite during the 3rd day. This distress continued with crampy pains in muscles of arms and legs even though the  $\text{NH}_4\text{Cl}$  was reduced to 7 gm.

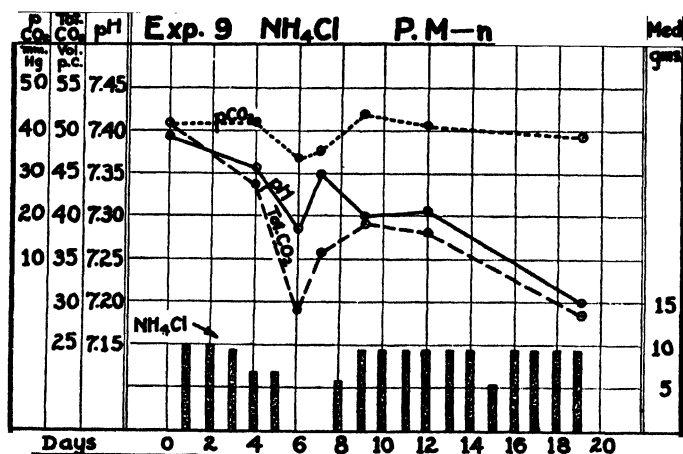


FIG. 8.

daily. On the 6th day the urine showed a moderate amount of albumin, a few red blood cells, and granular casts. The blood non-protein nitrogen was 68 mg. per 100 cc. No  $\text{NH}_4\text{Cl}$  was given during the 6th and 7th days. On the 7th day the non-protein nitrogen was 58 and on the 8th, 42 mg. per 100 cc., and the urine showed only an occasional granular cast. The blood pH at the end of the 6th day was still 7.28 and the total  $\text{CO}_2$  29.5 volumes per cent even though no  $\text{NH}_4\text{Cl}$  was given that day. The patient felt so well on the 8th day that  $\text{NH}_4\text{Cl}$  was again started in small amounts. There was no marked symptomatic disturbance, and the urine remained negative and the blood

non-protein nitrogen normal. The  $\text{NH}_4\text{Cl}$  was discontinued on the 19th day with a blood pH of 7.20 and a total  $\text{CO}_2$  of 28.2 volumes per cent.

The effect of  $\text{CaCl}_2$  is shown in Experiments 10 and 11, Fig. 9. The subjects were two apparently normal young men and the usual daily hospital work was continued during the experiment. A constant diet was maintained and fluids were limited to 2400 cc. daily. 15 gm. of  $\text{CaCl}_2$  were given daily, 5 gm. after each meal. At the end of the 8th day the discomfort became so great that work could no longer be continued and the  $\text{CaCl}_2$  ingestion was stopped.

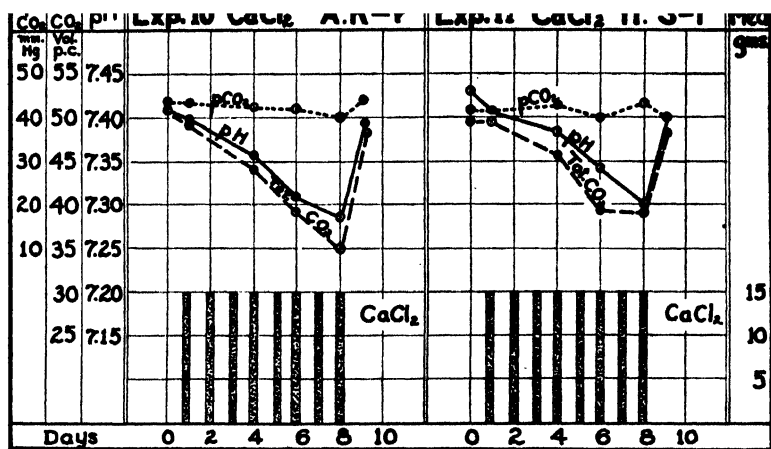


FIG. 9.

The distress consisted of ease of fatigue even upon slight exertion, general mental and physical lassitude and, in the case of H. S-I moderate headaches. During the first 2 to 3 days there was a definite diuresis but after this the urine output soon returned to normal.

#### *Administration of Alkali or Alkali-Producing Substances.*

The effect of  $\text{NaHCO}_3$  in the case of J. S-w is shown in Experiment 12, Fig. 10. At first 12 and later 20 gm. of  $\text{NaHCO}_3$  were given daily for 16 days. The blood total  $\text{CO}_2$  and pH increased in such a ratio that the  $\text{pCO}_2$  remained practically the same or

only slightly increased. The volume respiration increased slightly. During the course of the period the patient developed lassitude and lethargy, nausea, occasional headaches, and later general malaise with loss of appetite. Exercise consisting of ascending and descending a short flight of stairs produced marked fatigue, dizziness, nausea, and headache. The patient responded very poorly to partial anoxemia as compared to a similar test during the control period. A rebreathing spirometer of 18 liters capacity was used for this test and the patient became distressed at 11 per cent  $O_2$ , while formerly he could easily reach a much lower level. In spite of the

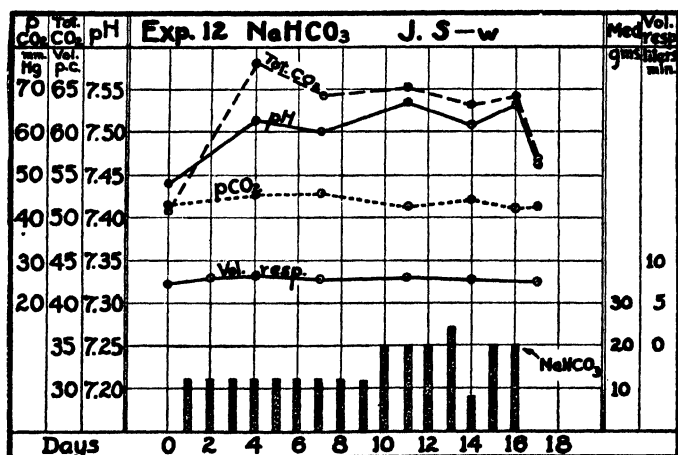


FIG. 10.

malaise and poor appetite, there was a gradual gain in weight of about 1.4 kilos.

The effect of large doses of NaHCO<sub>3</sub> in the case of J. T-a is shown in Experiment 13, Fig. 11. Approximately 40 gm. of NaHCO<sub>3</sub> were given daily for 21 days. The symptomatic response was very similar to that in the preceding experiment although twice the daily dose was given. There was only a moderate increase of 0.2 to 0.3 pH of the blood in the latter case in which the high alkali dosage was given. The response to exercise and partial anoxemia was also very poor in this case. The urine did not show albumin, casts, or cells at any time. After the alkali was stopped, the lassitude persisted for about 2 days.

The effect of approximately 40 gm.  $\text{NaHCO}_3$  a day, in the case of P. M-n, is shown in Experiment 14, Fig. 12. The response of

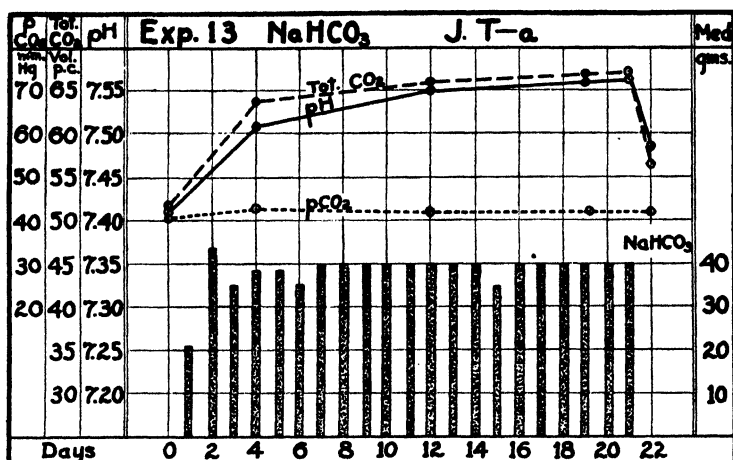


FIG. 11.

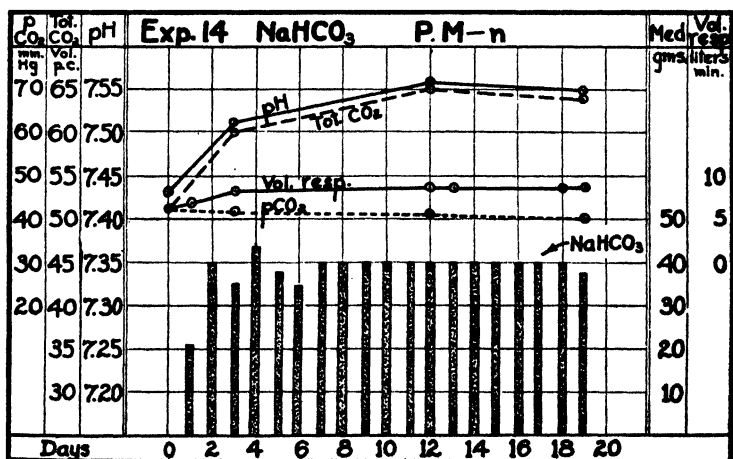


FIG. 12.

blood pH and total  $\text{CO}_2$  is quite similar to that in the preceding experiment. After the pH reaches a value of approximately 7.55, a level is maintained in that vicinity. In this case there was



practically no change in  $p\text{CO}_2$  but the volume respiration was definitely increased. At one time the patient showed a slight trace of albumin, granular casts, and a few epithelial cells and leucocytes but these disappeared in a few days without stopping the alkali. The patient complained of lassitude, drowsiness, and was quite listless and disinclined toward all activity after the first few days of alkali ingestion.

The effect of Na citrate in a dosage similar to that of  $\text{NaHCO}_3$  in the preceding experiments is shown in Experiment 15, Fig. 13. The subject, J. M-r, was a male Negro, approximately 35 years of

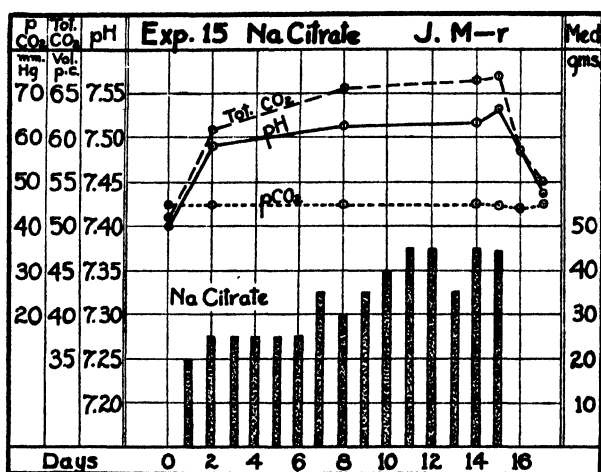


FIG. 13.

age. He had been under observation for about a week for the complaint of weakness and mild abdominal colic. There was no evidence of renal insufficiency. The hemoglobin was 80 per cent and the blood red cell count was 4,100,000. The response to Na citrate was quite comparable in all respects to that of  $\text{NaHCO}_3$ . The blood pH had increased to 7.53 and the total  $\text{CO}_2$  to 67 per cent. Lassitude and loss of appetite developed and the response to exercise and anoxemia was poor. Recovery from the blood changes and symptomatic distress was practically complete in 2 days.

## DISCUSSION.

That the pH of the blood can readily be changed by several tenths in either direction from the normal and maintained for several weeks without disastrous effect is demonstrated. Experiments 1 to 10 show that the pH can be lowered quite rapidly in 4 to 6 days from values of 7.45–7.40 to 7.30–7.25. After this the drop becomes more gradual until the low values of 7.20 to 7.15 are reached in 10 to 20 days of acid ingestion. The longest period of acidosis maintained was in Experiment 1, Fig. 1, where it extended to 25 days of  $\text{H}_3\text{PO}_4$ , and later also  $(\text{NH}_4)_2\text{HPO}_4$ , ingestion. During the latter 9 days of the experiment the blood pH was maintained in the vicinity of 7.20. The most severe acidosis, pH 7.17, was obtained after 16 days of  $\text{NH}_4\text{Cl}$  ingestion as shown in Fig. 6.  $\text{H}_3\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ , and  $\text{CaCl}_2$  can be employed successfully to produce definite lowering of the blood pH.  $\text{H}_3\text{PO}_4$  is quite unpleasant to take by mouth but it can be taken in amounts of 15 to 17 gm. daily.  $\text{NH}_4\text{Cl}$  is about twice as efficient for producing a grade of acidosis similar to that caused by an equal weight of  $\text{H}_3\text{PO}_4$ . 15 gm. of  $\text{NH}_4\text{Cl}$  can well be taken daily and will in approximately a week's time produce a pH lowering to 7.20 as shown in Experiments 7 and 8.

The measurement of blood pH during ingestion of alkali or alkali-producing substances shows that a real blood alkalosis can be produced and maintained.  $\text{NaHCO}_3$  ingestion in amounts of approximately 40 gm. daily produced a fairly rapid increase in blood pH from 7.40 to 7.44 to approximately 7.50 in 3 to 4 days, with a subsequent gradual rise to about 7.50 to 7.57 in 2 to 3 weeks (Experiments 12, 13, and 14). In Experiment 14, Fig. 12, the pH value remained at a level at 7.55 during the last week even though 40 gm. of  $\text{NaHCO}_3$  were administered daily. At this level the acid-base relationship had established a new balance with excretion of base equalling the intake.

Na citrate in amounts equal to the Na equivalent in  $\text{NaHCO}_3$  produces an effect on the blood pH practically equal to that of the latter as shown in Experiment 15.

*Total  $\text{CO}_2$ ,  $\text{CO}_2$  Tension, and Respiration.*

Haldane had previously shown that  $\text{NH}_4\text{Cl}$  taken in 15 to 20 gm. amounts for 3 days distinctly lowered the  $\text{CO}_2$  capacity of the

blood. He demonstrated a fall in the  $\text{CO}_2$  capacity from 48.1 to 28.2 volumes per cent with a simultaneous drop in the alveolar  $\text{CO}_2$  from 38.8 to 27.8 mm. Hg. An increased volume respiration from 6 to 10.4 liters per minute was observed in this case and Haldane concluded that 96 per cent of the acidosis was compensated by the increased respiration. On the other hand, Davies, Haldane, and Kennaway found no drop in the alveolar  $\text{CO}_2$  after the ingestion of 500 cc. of 0.116 N HCl or after 30 gm. of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ .

In all our apparently normal cases, acid or alkali ingestion caused a change in the total  $\text{CO}_2$  content of the blood that closely paralleled the pH change. The ratio of pH to  $\text{CO}_2$  content remained such that no gross changes occurred in the  $\text{CO}_2$  tension. However, there was a distinct tendency of the  $\text{CO}_2$  tension of the venous blood to fall in acid feeding but not to the magnitude that occurred in Haldane's arterial blood, judging from his alveolar  $\text{CO}_2$ .

In the one case where we obtained arterial blood during severe acidosis (Experiment 1, 22nd day), there was a more distinct fall in  $\text{pCO}_2$ , more comparable to that estimated from the increase in volume respiration. Not only is it natural for the changes in the venous  $\text{pCO}_2$  to be retarded over that of the arterial tension, but venous blood with a lowered bicarbonate content must necessarily have an increased  $\text{CO}_2$  pressure in order to carry away the  $\text{CO}_2$  from the tissues at the normal rate, assuming a constant blood flow. Therefore, it is perfectly possible that during a simple acidosis the venous blood has a normal  $\text{pCO}_2$  while that of the arterial blood is definitely decreased. Consequently, the  $\text{CO}_2$  tension of the tissues is probably still higher than that of the venous blood and a more marked tissue acidosis exists than either arterial or venous blood pH indicates.

The respiratory minute volume increased in all cases of acid feeding and in general varied inversely with the pH of the venous blood. The ventilation increased from approximately 5 liters per minute to 10 liters and in the more severe acidosis, pH 7.20, increased to 15 liters per minute. Haldane states in respect to his case of  $\text{NH}_4\text{Cl}$  acidosis: "When the alveolar  $\text{CO}_2$  fell, there was marked air-hunger. The increased frequency and depth of breathing was obvious. Thus at a time when the alveolar  $\text{CO}_2$  was 29.7 mm. the volume breathed per minute when sitting in a chair was 10.4 liters."

Our experience in this respect has been that with the patients lying quietly in bed the volume respiration could easily be doubled without any obvious increase by inspection. None of our patients while at rest showed any tendency toward air-hunger even though the blood pH was below 7.20, but only slight exertion would produce a marked dyspnea. In the case of J. L-r, Experiment 4, with a blood pH of 7.19, walking slowly across the room and back, a total of 45 feet, caused a moderate hyperpnea of 27.6 liters per minute, while later with a pH of 7.40, this same amount caused the ventilation to increase to only 13.2 liters per minute.

During the course of induced alkalosis the change in the total  $\text{CO}_2$  content of the venous blood paralleled that of the pH closely. Here too, as in the acidosis, the relationship remained such that the  $\text{pCO}_2$  remained nearly constant. In two of the four cases of alkalosis, there was a slight rise in the  $\text{pCO}_2$  for the 1st week. There seemed to be a slight increase in the volume respiration during the course of the alkalosis as previous workers have already observed (8). In the case of P. M-n, Experiment 14, the ventilation increased from 6 to approximately 8 liters per minute. The patients with alkalosis were all very sensitive to anoxemia. Ordinarily these subjects could easily lower the  $\text{O}_2$  content of a re-breathing spirometer from 20.8 per cent to 5 or 6 per cent in 7 to 8 minutes without objective symptoms, but the patient with alkalosis became dizzy, developed headaches, had visual and auditory disturbances before 10 per cent  $\text{O}_2$  was reached in the course of 4 to 5 minutes. In all probability, the patient in alkalosis is suffering from a mild anoxemia even at atmospheric  $\text{O}_2$  pressure. It is quite possible that this anoxemia is responsible for the increased volume of respiration during alkalosis following alkali ingestion. Not only does the alkalosis increase the affinity of the hemoglobin for  $\text{O}_2$  and thus diminish the supply to the tissues, but it probably depresses tissue oxidation also (9).

### *Symptomatology.*

It is now nearly 50 years since Walter's observations that administrations of large doses of dilute hydrochloric acid to rabbits produced listlessness, air-hunger, stupor, coma, and finally respiratory and cardiac failure. Since that time the association of these

symptoms with certain metabolic disorders that give rise to an acidosis have been well recognized clinically. However, in these cases where an acidosis exists as a secondary factor based upon some primary pathological condition, it has always been a problem as to how much of the symptomatology was due to the acidosis and how much was due to other derangements.

For this reason the general symptomatology of these patients in whom a simple acidosis was produced for a considerable period by acid feeding, was carefully noted. The distress that occurred in most cases, if acid intoxication was carried sufficiently far, developed approximately in the following order: loss of appetite, lassitude, listlessness, headaches, weakness, nausea, occasionally vomiting, symptoms of dehydration, muscular aches and pains and abdominal cramps, loss of weight, hyperpnea, drowsiness, and renal insufficiency. The order is, of course, approximate only, for often the several different types of distress may come on at the same time and in some cases the sequence may be considerably changed.

*Loss of Appetite.*—This condition developed in all of the eleven acidosis cases and usually came on during the first few days of mild acidosis and then improved, but later returned more markedly as the acidosis became severe. The first period of anorexia was probably more related to the disagreeableness of taking the acids or salts by mouth and the resulting temporary purgation, than to the acidosis itself. Adjustment then seemed to take place and for a period of a week to 10 days the appetite was fairly good, even though the blood pH gradually decreased from 7.30 to below 7.25. Finally, however, with the general marked depression, the appetite failed completely and all food was refused.

*Lassitude, Listlessness, Drowsiness, and Stupor.*—In general, as the blood pH dropped from normal to 7.30 or lower in the course of the first 3 or 4 days, the subject gradually became disinclined toward any activity and lost interest in his surroundings. This condition gradually increased as the acidosis progressed. In general, the acid medication was stopped before this distress became marked but in a few cases the lassitude and listlessness progressed to definite drowsiness as the blood pH dropped below 7.20 after being low for a period of a week or 10 days. In several of these cases mental dullness, especially the inability to concentrate,

became very pronounced. This was especially noticeable in the cases of A. K-r and H. S-l (Fig. 9) where an attempt was made to carry on mental work each day.

*Headaches.*—Generalized, dull headaches occurred in a majority of the subjects with severe acidosis although the occurrence was usually inconstant and irregular. Any slight effort usually initiated a headache and as the acidosis progressed this distress would usually come on spontaneously and persist for days. The headaches were probably a secondary result from the general debility, although a cerebral edema secondary to depressed oxidation might also have played a rôle.

*Weakness.*—Fatigability and weakness are usually noticed after 3 or 4 days of mild acidosis and gradually increase in severity as the acidosis progresses. The ease of fatigue is more pronounced than the loss of strength. This is well exemplified by the observation that normal strength may be exhibited in flexion of the forearm but if this movement is repeated several times marked fatigue develops. This probably is due to the development of a still more severe tissue acidosis due to  $\text{CO}_2$  accumulation as a result of the poor  $\text{CO}_2$ -carrying power of the blood. Loss of muscular power as well as endurance, however, develops as the acidosis becomes severe and prolonged.

*Dehydration.*—In practically all cases it was noticed that as the acidosis progressed, it was more and more difficult to obtain blood samples from the veins of the cubital region of the arm. Not only did the skin and subcutaneous tissue seem to lose their turgor and normal consistency, but the veins seemed definitely less prominent. Dryness of the mucous membranes could be noted and the patients frequently complained of dryness of the mouth. There was also a distinct loss of weight amounting usually to 2 to 3 kilos during the period of the acidosis, the greater portion of which usually was lost during the first 4 or 5 days. This period of rapid loss usually was coincident with that of the initial diuresis and purgation. If reduced food intake or autolysis of tissue proteins due to decreased pH were the cause of weight loss, it would be expected that the greater loss would occur during the latter period of severe acidosis. The loss in weight during  $\text{CaCl}_2$  ingestion was first noted by Blum (10) and his coworkers who used this salt successfully in the treatment of edema. They also

noted a distinct concentration of the plasma proteins during the first few days when diuresis occurred. These workers attributed the loss of water to the effect of calcium but Haldane, Hill, and Luck (5) showed that a similar dehydration occurred when  $\text{NH}_4\text{Cl}$  was ingested. The explanation of these latter workers that the loss of water is due to the acidosis itself is probably correct. They state: "The increased acidity brings the blood and tissue proteins nearer to their isoelectric points, and they therefore release cations which they are holding in Donnan equilibrium, and diminish their osmotic pressure, thus losing water."

The introduction of the Donnan equilibrium is not clear, for the effect is probably one of decreasing protein dissociation and therefore the ability to unite chemically with cations, but the end-result as far as dehydration is concerned is the same. Later it will be shown that alkalosis has the opposite, that is, hydrating effect.

*Muscular Aches.*—Haldane and his coworkers (5) noted that  $\text{CaCl}_2$  produced great general discomfort, pains in the head, limbs, and back. They state that these effects never occur with  $\text{NH}_4\text{Cl}$  and must be attributed to the calcium. In our experience, these muscular aches and pains came on in a majority of our severe acidosis cases, whether caused by  $\text{CaCl}_2$ ,  $\text{NH}_4\text{Cl}$ , or  $\text{H}_3\text{PO}_4$  ingestion. Exertion seemed especially to be a predisposing factor in bringing on these pains and in one case severe aches and spasm resulted in the muscles of the legs after slight exertion. These were similar to the ordinary muscle cramps occasionally experienced after severe exercise. In both cases the condition is probably due to the acidosis or its secondary effect upon metabolism.

*Air-Hunger.*—This condition, usually associated by the clinician with acidosis, was not present in any of these cases in the degree of acidosis obtained while the patient was at complete rest. Slight exertion, however, usually brought it on. Clinically, of course, more severe acidoses are seen, occasionally below pH 7.00 and air-hunger then may be a striking feature.

*Renal Insufficiency.*—Only one of our cases developed temporary renal insufficiency (P. M-n, Experiment 9) and we have seen several more since where no renal incompetency was apparent before. In all these cases  $\text{NH}_4\text{Cl}$  was administered and the question arises whether the extra amount of ammonia to be excreted was a factor in renal impairment and nitrogen retention.

*Symptomatology During Alkalosis.*—That the symptoms of acidosis and alkalosis may be quite similar is now being generally appreciated. In our study, practically every symptom elicited during acidosis was also noted during alkalosis; namely, loss of appetite, lassitude, listlessness, headaches, weakness, nausea, and drowsiness. Renal insufficiency, especially where some impairment existed previously, has been reported in the literature (11). Air-hunger, stupor, and irrational mental states were of course not observed in the cases reported here but these conditions have been observed where the diagnosis of alkalosis was definitely established and disappeared when the blood reaction was restored to normal (12). Symptoms of dehydration, however, are not present in alkalosis as contrasted with acidosis, in fact, there usually is definite evidence of hydration. The edema developing after excessive  $\text{NaHCO}_3$  administration so frequently observed is an example of this disturbance in the water balance. All of our patients, whether given  $\text{NaHCO}_3$  or Na citrate, showed an increase in weight from 0.90 to 3.3 kilos. In the case of P. M-n, Experiment 14, where there was only a slight increase and finally a loss in weight, his general condition was poor and there was marked anorexia. Undoubtedly poor nutrition was a definite factor in counteracting the apparent effect of hydration as far as weight was concerned. Although the edema of  $\text{NaHCO}_3$  ingestion has been looked upon as due to Na retention, the primary effect is probably related to the increased pH and its influence on the protein-salt osmotic effect, as described under dehydration in acidosis. It is logical to assume that the same reason that causes dehydration in acidosis causes hydration in alkalosis.

That the subject with alkalosis is especially sensitive to anoxemia has already been mentioned. His susceptibility to fatigue is markedly increased and exercise increases most of his distress, especially headache and lassitude. In general it would be expected that the acids liberated during exercise would diminish the alkalosis and the accompanying distress. This neutralizing effect cannot be appreciable, especially in view of the large alkali reserve, and it must be assumed that the alkalosis has a depressing effect on the metabolism of exercise.



*Relation of Acidosis and Alkalosis to General Metabolism.*

It is readily seen that the general well being and functional activity is at its optimum at the normal reaction of the body and that a depression occurs with a variation of the pH in either direction. Restlessness, lassitude, debility, fatigability, headaches, listlessness, and finally stupor are symptomatic reactions of both acidosis and alkalosis. These are, however, also the usual reactions associated with depression of the energy metabolism as in chronic anoxemia as obtained at high altitudes, carbon monoxide poisoning, methemoglobinemia, cyanide poisoning, marked anemia, hypothyroidism, etc. In earlier work we have already shown that in death by anoxemia, life can be greatly prolonged by maintaining a normal reaction of the blood; that variation in either the direction of acidosis or alkalosis shortens life (13). It has also been shown that in tissue oxidation *in vitro*, the rate of oxygen consumption apparently is at its optimum at approximately the normal blood reaction with a depression in either direction. It is possible that the ill effects of acidosis and alkalosis are produced through the common channel of depression of tissue oxidation. This conception would explain the similar symptomatic effects of two apparently separate and opposite conditions.

It is a great pleasure to express my appreciation for the kind help and facilities extended to the writer during this work by Drs. J. H. Means, J. C. Aub, and A. V. Bock of the Massachusetts General Hospital.

## SUMMARY.

An acidosis of blood pH 7.30 to 7.20 can readily be produced and maintained for a period of several weeks by the ingestion of  $\text{H}_2\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{CaCl}_2$ ,  $(\text{NH}_4)_2\text{HPO}_4$  or combinations of these substances.

An alkalosis of blood pH 7.50 to 7.55 can be maintained for several weeks by the administration of  $\text{NaHCO}_3$  or Na citrate.

In normal individuals recovery from this grade of acidosis or alkalosis is rapid and usually complete in 24 to 48 hours.

The symptomatic distress during acidosis or alkalosis is similar; namely, lassitude, nausea and later vomiting, anorexia, headaches,

weakness, listlessness, muscle aches, and drowsiness. A case of renal involvement was noted both in acidosis and alkalosis. Opposite effects apparently are obtained in regard to weight and hydration; during acidosis there is decrease in weight with signs of dehydration, while during alkalosis there is evidence of hydration with increase in weight. There seems to be a slight increase in ventilation during alkalosis.

The change in total venous blood  $\text{CO}_2$  during acid or alkali ingestion parallels that of blood pH closely as long as there is no renal involvement or respiratory upset.

The  $\text{CO}_2$  tension of the venous blood has a tendency to decrease slightly during acid ingestion but in general does not change markedly, while the  $\text{CO}_2$  tension of the arterial blood drops quite appreciably. During alkali ingestion the venous blood  $\text{CO}_2$  tension does not change to any extent.

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## NON-PROTEIN SULFUR COMPOUNDS OF BLOOD.

## I. SYMPECTOTHION.\*

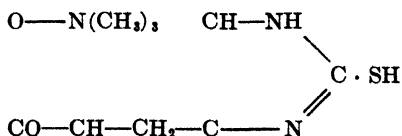
BY GEORGE HUNTER AND BLYTHE ALFRED EAGLES.

(From the Department of Pathological Chemistry, University of Toronto,  
Toronto, Canada.)

(Received for publication, June 23, 1926.)

We have previously reported (4) the isolation from blood corpuscles of a compound believed to be mainly responsible for the discrepancies in certain colorimetric methods for the estimation of uric acid. In a previous paper by Bulmer, Eagles, and Hunter (2) the interfering substance shown to be present was provisionally called substance X and this term was continued in the later paper by the present writers in the hope that an early elucidation of its chemical structure would render unnecessary a non-chemical name. The substance, however, has since proved to be more complex than we anticipated and on that account a suitable name for it now seems desirable. A notable property of the substance is that when boiled with concentrated sodium hydroxide and lead acetate there is no formation of lead sulfide<sup>1</sup> as there is when glutathione, which we show later to be another blood constituent, is

\* Since this paper was submitted for publication Eagles and Johnson (8), to whom I sent its galley proof, have shown that sympectothion is identical with the ergothioneine of Tanret (9), represented by the formula  $C_9H_{18}N_2SO_2$ , and shown by Barger and Ewins (10) to be the betaine of thiolhistidine.



It will thus be seen that the formula  $C_{13}H_{22}N_6S_2O_5$  here given for sym-pectothion is twice that of ergothioneine plus 1 molecule of water.—G. H.

<sup>1</sup> Corrections: It was erroneously stated in our previous paper that our new substance contained no sulfur. Through an oversight the fusion test

similarly treated. In these two non-protein sulfur constituents of blood we have examples of the classical distinction suggested by Krüger (6) of "firmly bound" and "loosely bound" sulfur respectively. The terms have been used in the literature almost since their introduction so that it seems not inappropriate to utilize this idea in the present instance. We thus suggest that the substance be known as *sympectothion* (Greek *συμπηκτός* = firmly bound, *θείον* = sulfur<sup>2</sup>).

The formula previously submitted for sympectothion was  $C_6H_{11}N_2O_3$ <sup>1</sup> and that now submitted is  $C_{13}H_{32}N_6S_2O_5$ . The revised formula is thus thrice the original with 4 oxygen atoms replaced by 2 sulfur atoms and less 1 atom of hydrogen.

It is desirable at this stage to consider the relationship of sympectothion to a compound described by Benedict, Newton, and Behre (1) since the appearance of our last paper and named by them "thiasine." The properties of thiasine are in so many respects similar to those of sympectothion that readers of the two papers may think that the two substances are identical. And it is indeed to allow for the possibility that the two substances may ultimately prove to be the same substance, originally described by us as substance X (4), that it is desirable to consider their relationship at this point in our paper.

The properties of thiasine are in striking accord with those of substance X previously described, and yet the discrepancies are so great that Benedict, Newton, and Behre come to the conclusion that "assuming an error in regard to the absence of sulfur and direction of rotation of 'substance X,' it is difficult to reconcile the reported findings for the elementary composition of this substance with those which would be yielded by a reasonably pure sample of thiasine." Benedict, Newton, and Behre have not given us any further information by which we might judge differently.

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with sodium had at that time not been applied to the substance. Alongside this substance, by a scheme outlined in a subsequent paper, we prepared glutathione, and frequently used the lead acetate and sodium hydroxide test to distinguish the compounds. From Tunnicliffe's (7) finding that the sulfur of glutathione accounted for almost all of the organic sulfur in tissue extracts we were further misled. Our paper had however just left the press before we discovered our error and no correction could then be made.

<sup>2</sup> I am indebted to Prof. George O. Smith of University College for suggesting the name. G. H.

The two substances may be compared and contrasted. Thus, qualitatively, we found in common with Benedict, Newton, and Behre that the substance is precipitated by silver nitrate, mercury chloride, and by picric acid from relatively concentrated solution; that it gives a positive Weyl's test, a blue color with the uric acid reagents, but a negative test with the qualitative reagents for sugars; that the substance contains no primary amino nitrogen and that it is very resistant to the action of strong acids. The m. p. of the substance obtained by Benedict, Newton, and Behre is  $262\text{--}263^\circ$  as compared with  $269\text{--}270^\circ$  previously reported by us.

The properties of the substance described by Benedict, Newton, and Behre, which had not already been reported by us, are the presence of sulfur in the substance, the finding that it forms a hydrochloride, and the finding of a specific rotation of the same magnitude but of the opposite sign from that previously given by us.

The two reports on the qualitative tests are thus surprisingly confirmatory, and in some quantitative measurements the agreement is remarkable. The specific rotations are identical,  $+116^\circ$  for thiasine and  $+115^\circ$  for sympectothion (by error reported as  $-115^\circ$  in our former paper). The color intensities with Folin's uric acid reagent are likewise identical. Thus it was found by Benedict, Newton, and Behre, when using the cyanide method, that 1 part of uric acid gives the same amount of color as 7.5 parts of their substance, and when using the carbonate method 1 part of uric acid gives the same amount of color as 1.6 parts of their substance. We had previously reported the corresponding uric acid color equivalents of our substance by the two methods as 7.5 and 1.5 parts respectively.

The failure to identify the substance isolated by Benedict, Newton, and Behre with sympectothion thus rests principally on the disagreement in the elementary analyses. The formula obtained by Benedict, Newton, and Behre is  $\text{C}_{12}\text{H}_{20}\text{N}_4\text{O}_3\text{S}$  as compared with that given in the earlier part of this paper  $\text{C}_{13}\text{H}_{22}\text{N}_6\text{S}_2\text{O}_5$ . This indicates a difference in carbon of 2.62 per cent, in hydrogen of 0.12 per cent, in nitrogen of 0.95 per cent, in sulfur of 2.84 per cent, and in oxygen of 0.39 per cent.

From the foregoing analysis it is obvious that in the present stage of the research sympectothion cannot be identified with

thiasine; so that our only course is to consider them two separate substances and leave their exact relationship to future work to determine.

It is, however, desirable in the circumstances to give the results of analyses performed subsequent to the appearance of the paper of Benedict, Newton, and Behre as well as our values for sulfur. We have to thank Professor L. J. Rogers of the Department of Analytical Chemistry for the carbon and hydrogen values.

*Nitrogen.*

0.1023 gm. of substance yielded	17.52	per cent nitrogen.
0.0764 " " " " "	17.51	" " "
Micro-Kjeldahl, 9.2 mg. " " "	17.61	" " "

*Carbon and Hydrogen.*

Substance. gm.	CO <sub>2</sub> gm.	H <sub>2</sub> O gm.
0.1800	0.3064	0.1141
0.2852	0.4700	0.1560
C	H	
per cent	per cent	
46.42	7.04	
44.95	6.08	
Average. 45.68	6.56	

*Sulfur* was determined by both fusion and Carius methods. The values obtained on material from three separate batches of blood are:

1. 0.0996 gm. of substance yielded	13.36	per cent sulfur.
2. 0.1046 " " " " "	13.18	" " "
3. 0.1060 " " " " "	13.19	" " "

Average. 13.24

Taking our previously published results for *carbon*, *hydrogen*, and *nitrogen* we then have:

	C <sub>15</sub> H <sub>22</sub> N <sub>8</sub> S <sub>2</sub> O <sub>6</sub> . Calculated.	Found.
C.....	45.38	45.38
H.....	6.72	6.75
N.....	17.65	17.54
S.....	13.44	13.24
O.....	16.81	17.09

The minimum molecular weight of the substance is thus 476. From our previous results it would appear that the substance behaves abnormally in depressing the freezing point in aqueous solution.

*Specific Rotation.*—Through a slip in our previous paper the rotation,  $[\alpha]_D^{27.5^\circ}$ , was given as  $-115.0^\circ$ . The substance is dextro-rotatory and  $[\alpha]_D^{27.5^\circ}$  is  $+115.0^\circ$ .

*Other Properties.*—Sympectothion is soluble in water at ordinary temperature to the extent of about 20 parts per 100 parts of water. It is very soluble in hot water and recrystallizes as colorless needle-shaped crystals when hot concentrated solutions are cooled.

When water is added drop by drop to the dry substance until it is just wet it assumes a somewhat curdy appearance with little sign of dissolving and then dissolves very rapidly with a few more drops of water.

When the substance is mixed with sodium peroxide and potassium hydroxide, for the estimation of sulfur, a marked odor of trimethylamine is perceptible even in the cold. When fused with potassium hydroxide the same odor is produced.

When sympectothion is boiled for 9 hours with 20 per cent hydrochloric acid no free amino nitrogen, according to the Van Slyke method, is produced.

#### *Modifications in the Preparation of Sympectothion.*

The method previously described for the isolation of sympectothion can be simplified in some minor respects without affecting the purity of the end-product. In our recent preparations we have found it more convenient to use whole blood than centrifuged corpuscles, although it is desirable to use corpuscles if facilities for easy centrifugation are available, as the protein coagulated with sulfuric acid and heat is somewhat firmer and more easily manipulated in the case of corpuscles than in the case of whole blood. Nevertheless a very satisfactory coagulum is obtained from *fresh* whole blood mixed with half its volume of water and three-eighths its volume of 0.1 normal sulfuric acid when heated to about  $80^\circ$ . Without the addition of any acetic acid as previously advised the cooled filtrate is then precipitated with uranium acetate as before.



The glutathione fraction<sup>3</sup> is then removed with basic lead acetate known as "Goulard's extract" (3). We have found very constantly that about 0.13 cc. of this lead acetate solution is required for each 10 cc. of uranium acetate filtrate to free the supernatant from glutathione and still leave the reaction acid to litmus paper.

As previously described the sympectothion is then precipitated with mercury chloride solution, the washed mercury compound freed with hydrogen sulfide, and the solution freed from mercury sulfide by centrifugation and from hydrogen sulfide by aeration.

The filtrate amounting to about 600 cc. is nearly neutralized with 2.5 normal sodium hydroxide, and when using 12 to 14 liters of blood, 300 cc. of 20 per cent aqueous sugar of lead are added. The sandy precipitate which readily settles and which consists partly of the lead compound of glutathione,<sup>3</sup> is filtered off and the filtrate made to a definite volume (1000 cc.).

In our previous paper we considered the next step, the precipitation of sympectothion by the addition of sodium hydroxide, as important in the fractionation, but remarked that it was not unattended by difficulty. The difficulty is however readily overcome by the addition of sodium chloride after the requisite amount of sodium hydroxide has been added. Thus, a 10 cc. aliquot portion of the fluid is taken in a centrifuge tube and 2.5 normal sodium hydroxide added in 0.1 cc. portions until the color of the mixed contents turns to a faint creamy yellow. There is then added 0.5 cc. of 10 per cent sodium chloride solution and the tube is inverted to mix. If the required amount of sodium hydroxide has been added 1 cc. of the clear centrifugate will give no color with phosphotungstic acid and sodium carbonate. If too little sodium hydroxide has been added the supernatant liquid will give a blue color with the uric acid reagents, a further precipitate on the addition of sodium hydroxide, and an excess of lead will show its presence by a cloudiness produced when the phosphotungstic acid reagent is added to the test portion. If there is no cloudiness here, and if the solution gives a blue color, too much sodium hydroxide has been used and the supernatant liquid will

<sup>3</sup> See following paper, Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 133.

give a precipitate on the addition of a further small amount of lead acetate solution. In the presence of 0.5 per cent of sodium chloride the test solution readily centrifuges to give a clear supernatant liquid so that there is no difficulty in obtaining suitable test portions. We find when working with about 13 liters of blood, when 300 cc. of 20 per cent lead acetate have been used and when the volume of the filtrate is 1000 cc., that a total of about 90 cc. of sodium hydroxide with 50 cc. of sodium chloride quantitatively frees the solution of sympectothion. But a slight variation in the concentration of sodium hydroxide in either direction leaves some of the substance in the supernatant liquid, so that when near the end-point in the test solutions the amount of sodium hydroxide should be varied by not more than 0.02 cc. With a little practice the exact amount of sodium hydroxide to be added can be determined with two to four 10 cc. test portions.

The lead precipitate obtained is centrifuged, washed once with 0.5 per cent solution of sodium chloride, and again with its own volume of water. The substance is freed from lead by grinding in a mortar with 0.2 normal sulfuric acid until the filtrate from a test portion shows the presence of excess of the acid.

The precipitation of the filtrate with the Hopkins and Cole mercury sulfate reagent as in our original method appears to be unnecessary, so we now omit this step. Indeed in our subsequent work we occasionally found some difficulty in getting a well coagulated precipitate without the addition of a large excess of the reagent and the addition of an undesirable amount of sodium hydroxide.

The filtrate from the lead sulfate, amounting to about 500 cc., is accordingly neutralized with sodium hydroxide, and sulfuric acid added to 0.5 normal. Phosphotungstic acid is added until precipitation ceases and the supernatant liquid shows practically no blue color with the uric acid reagents. The phosphotungstate is thoroughly washed and the remainder of the treatment followed as we have previously described.

The preparation thus described offers no difficulties and the substance may be obtained in pure form from 10 to 15 liters of blood in the course of 2 to 3 days. From numerous preparations we have never failed to obtain the substance readily in the crystalline form previously depicted by us.

An idea of the ease of preparation of sympectothion when the glutathione fraction is discarded, is perhaps obtainable from the fact that in the course of 3 weeks we were able to obtain 6.1 gm. of the pure substance from 117 liters of blood.

*Preparation without the Use of Hydrogen Sulfide.*

Having in mind especially the work of Johnson and Burnham (5) on the formation of thiopolypeptide derivatives by the action of hydrogen sulfide on aminoacetonitrile and the suggestion of Benedict, Newton, and Behre (1), it seemed desirable if possible to prepare sympectothion without the use of hydrogen sulfide, lest the sulfur were thereby introduced into the molecule. Such a preparation indeed proved to be more simple than we anticipated, and the method may be adopted in place of that which we have just described.

From 13.5 liters of blood the protein and glutathione were removed as we have outlined. The filtrate which amounted to 27,750 cc. was then precipitated by additional lead acetate and sodium hydroxide in a manner similar to that which we have described for the filtrate obtained from the mercury chloride fraction. Thus it was found that the addition of 0.20 cc. of 20 per cent sugar of lead and 0.135 cc. of 2.5 normal sodium hydroxide removed completely the color-giving substance from a 10 cc. test portion. The precipitate also coagulated readily without the addition of sodium chloride owing presumably to this and other salts in the solution. We then added to the total filtrate 555 cc. of the sugar of lead solution and 375 cc. of the sodium hydroxide. After allowing to settle for about 30 minutes the supernatant liquid was siphoned off and the remainder centrifuged. The washed precipitate was then freed with sulfuric acid in the usual way, the filtrate from the lead sulfate amounting to 1200 cc. This was made 0.5 normal acid with sulfuric acid and completely precipitated with about 50 cc. of 20 per cent phosphotungstic acid in 0.5 normal sulfuric acid and allowed to stand overnight in an ice chest. There was a rather small salmon-colored precipitate on the bottom and a light white precipitate on top. The white precipitate poured almost completely off with the supernatant leaving the heavy pinkish precipitate behind. The residue was centrifuged, thoroughly washed, and sympectothion recovered in the usual way.

The crystalline precipitate obtained on the addition of alcohol showed the characteristic crystals already pictured by us. 0.75 gm. of the pure dry substance was thus obtained, which gave a m.p. of 273–274° when heated over a period of 4 minutes.<sup>4</sup>

N.	Found.	17.64.	Calculated.	17.65.
S.	"	12.97.	"	13.44.

It is thus proved that sulfur is not introduced into the substance in the course of the preparation.

### *Human Blood.*

There is no reason to suppose that sympectothion is not present in human blood, as indicated by the color test which we have previously described. Nevertheless it is desirable to have some confirmatory evidence, since, as we show in our next paper, reduced glutathione also gives a blue color with the uric acid reagents.

About 1 liter of blood, obtained from children<sup>5</sup> during the exsanguination-transfusion operation, was treated as we have already described and the sympectothion fraction followed as far as the phosphotungstic acid precipitate. A characteristic pinkish phosphotungstate was obtained but the amount was too small, as would be expected, to permit of the isolation of the free substance. The evidence from the fractionation is however almost as good as the isolation of the substance itself, unless it is obtained in sufficient amount to permit at least of the estimation of nitrogen and sulfur.

### *Is Sympectothion Confined to Blood?*

It is yet too early to answer this question at all satisfactorily but in the course of our preparations of glutathione from liver and yeast we found that nearly all of the substance giving a color with the uric acid reagents was precipitated in the glutathione fraction by lead acetate in nearly neutral solution. This finding indicates that sympectothion is probably absent from yeast and liver.

<sup>4</sup> The m.p. of sympectothion varies over 2–3° according to the rate of heating. When heated over the same period we have found three other preparations from different batches to melt at 274–275°; 274–275°; 275–276°.

<sup>5</sup> We obtained this blood through the kind cooperation of Dr. T. G. H. Drake of the Hospital for Sick Children.

## SUMMARY.

For the substance previously isolated by us and then termed substance X we now suggest the name sympectothion, as it contains "firmly bound" sulfur. The formula previously submitted  $C_6H_{11}N_2O_3$  is now replaced by  $C_{18}H_{32}N_6S_2O_5$ .

Sympectothion has been prepared without the use of hydrogen sulfide so that the possibility of the introduction of sulfur in the course of the preparation is precluded.

The characteristic phosphotungstate of sympectothion has been isolated from human blood.

Certain simplifications of our previously described method for the isolation of sympectothion are given.

The relation to sympectothion of a substance recently isolated by Benedict, Newton, and Behre is discussed.

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## NON-PROTEIN SULFUR COMPOUNDS OF BLOOD.

### II. GLUTATHIONE.

BY GEORGE HUNTER AND BLYTHE ALFRED EAGLES.

*(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)*

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Two recent papers (2, 9) from this laboratory have shown that protein-free blood filtrates contain a substance, or substances, other than uric acid, which gives a blue color with the phosphotungstic acid reagent and alkali as ordinarily used for the estimation of uric acid. In one of the papers (9) we reported the isolation from pig blood of two non-protein substances both of which give a blue color with the uric acid reagents. A revised report on one of the substances, previously called substance X but now referred to as sympectothion, is furnished in the preceding paper.

The other substance, of which only mention was made in our previous paper and which was actually isolated before sympectothion, we suspected to be glutathione.

It had been stated by Arnold (1) that protein-free blood corpuscle filtrates give a positive test with sodium nitroprusside and ammonia indicating, according to this worker, the presence of cysteine. It had further been shown by Folin and Looney (5) that cysteine gives a blue color with the phosphotungstic acid reagent in presence of alkali. We had, of course, confirmed Arnold's finding of a positive nitroprusside test in protein-free blood filtrates and we supposed that the nitroprusside-reducing substance in blood filtrates was the same as the phosphotungstic acid-reducing substance. In view of Hopkins' finding (8) that glutathione was present in yeast, liver, and muscle it seemed probable that the sulfydryl group of glutathione was responsible for the discrepancies found in the estimation of uric acid and for the additional blue color given by phosphotungstic acid and sodium carbonate as used by certain French workers (cited in (9)).

We thus sought to account for the blue color given by the uric acid reagents by an attempt to isolate glutathione from blood corpuscles. The fractionation was followed by the two color tests, for it was reasoned that if glutathione were the interfering substance, the findings from the two tests would run parallel. We soon found, however, that they did not. In the neutral lead acetate precipitation of the uranium acetate filtrate we found that nearly all of the nitroprusside-reducing substance was removed but apparently only a fraction of the phosphotungstic acid-reducing substance. When the glutathione fraction was precipitated with phosphotungstic acid we obtained in one instance a small brick-red precipitate which, when freed with barium hydroxide, gave a filtrate which yielded only a faintly positive test with nitroprusside and a disproportionately strong test with the uric acid reagents. We were thus led to the conclusion that there are two non-protein substances in corpuscle filtrates which give a blue color with the phosphotungstic acid reagents. One of these we show here to be glutathione and the other we have called sympectothion.

We have appended a scheme of preparation for the isolation of the two substances in pure form from the same blood.

After we had actually obtained a small amount, about 200 mg., of glutathione from blood, a paper by Tunncliffe (11) appeared in which it was stated that glutathione was "completely absent" from rat, rabbit, and human whole blood. We thought it possible that Tunncliffe had other means of accounting for the positive nitroprusside test found by Arnold, although he offered no explanation. Tunncliffe's statement however made us reluctant to pronounce on the subject. There was the further difficulty that, as blood has an insignificant demand for oxygen, we should not expect to find in it, in relatively large amount, a substance which tends to be associated with an active metabolism. This is, of course, a teleological argument but nevertheless it is of the same order as that which attributes metabolic activity of any other tissue to the presence of glutathione in it.

Meanwhile, Holden (7) claimed to have isolated glutathione from sheep corpuscles. There is little doubt but that Holden's product contained some glutathione, yet it seems to us very improbable that his material was pure. From Holden's description it is almost certain that his glutathione was contaminated with

sympectothion. Contrary to Hopkins' procedure he used phosphotungstic acid to *precipitate* the glutathione, on the strength of Hopkins' statement that glutathione is precipitated in concentrated solution by phosphotungstic acid. But it is obvious that Holden could not have had a highly concentrated solution of glutathione in a volume of 50 cc. since he used only 4 liters of sheep blood for the preparation. It is more probable that some of Holden's material was oxidized before it reached the phosphotungstic acid precipitation, as oxidized glutathione like cystine is precipitable by phosphotungstic acid. And in omitting the subsequent copper hydroxide precipitation it seems inevitable that Holden obtained in his product as much sympectothion as glutathione. The only test of value applied by Holden to his product was that of the isolation of glutamic acid after hydrolysis, although he offers no proof that the crystals obtained were glutamic acid.

Indeed it would appear from a critical examination of Holden's paper that his evidence for the presence of glutathione in blood is little better than the evidence furnished by the positive nitroprusside test alone.

As the method described by Holden for the preparation of glutathione from blood appears to us to be unsatisfactory it is necessary to describe our own method somewhat in detail.

#### EXPERIMENTAL.

*Method of Preparation.*—Pig blood in batches of 10 to 13 liters was centrifuged to obtain the corpuscles and the latter freed of protein by heating with sulfuric acid and subsequent precipitation with uranium acetate as we have already described for the preparation of sympectothion.

In our earlier work we used neutral lead acetate for the next precipitation but later employed the basic lead acetate known as Goulard's extract (6). A 10 cc. aliquot portion of the uranium acetate filtrate, amounting generally to 20 to 25 liters, was tested with the basic lead acetate solution. It was generally found that about 0.13 cc. of this reagent was required to render the filtrate negative to the nitroprusside test, the solution still remaining faintly acid. The calculated amount of lead acetate was added to the filtrate, the precipitate allowed to settle, and the supernatant





liquid siphoned off. The suspended precipitate was centrifuged and washed twice in the centrifuge tubes with about twice its bulk of water. The washed precipitate was removed to a large mortar and ground to a thin paste with water. 2 normal sulfuric acid was added until a centrifuged specimen of the fluid just showed the presence of excess of sulfuric acid when tested with barium chloride. The mixture was filtered and the precipitate well washed with water.

To the yellow filtrate hot saturated barium hydroxide solution was added until precipitation just ceased. The mixture was then quickly centrifuged, the supernatant liquid poured off, and nearly neutralized with 2 normal sulfuric acid. Without filtration of the barium sulfate, the Hopkins and Cole mercury sulfate reagent was added until precipitation ceased. The washings from the barium hydroxide precipitate were treated similarly.

The mercury precipitate was centrifuged and washed well with water in the centrifuge tubes. It was suspended in about 80 cc. of water and the mercury freed with hydrogen sulfide.

The sulfide-free filtrate, amounting generally to about 300 cc., was again precipitated with the mercury sulfate reagent, and the sulfide-free filtrate from this, amounting generally to about 150 cc., was precipitated with freshly precipitated copper hydroxide as described by Hopkins for yeast. The copper-glutathione precipitate was washed in the centrifuge tubes four times with about twice its bulk of water. The copper was then removed as sulfide and hydrogen sulfide removed from the filtrate by a current of air washed successively with sodium hydroxide and sulfuric acid. When most of the hydrogen sulfide had been removed a few drops of 2 normal sulfuric acid were added and aeration continued for 2 to 3 hours to insure complete removal.

The solution was adjusted to a pH of about 7.5 with barium hydroxide and aeration continued until a test portion no longer showed a positive test with sodium nitroprusside and ammonia or phosphotungstic acid and sodium carbonate. Sulfuric acid and barium hydroxide were quantitatively removed from the solution, which was concentrated *in vacuo* to a thin syrup, and about 10 volumes of absolute alcohol added to precipitate the oxidized glutathione. After standing overnight the friable mass was ground, filtered, and finally washed with absolute alcohol. We

have rarely been able to obtain more than 0.5 gm. of the dried material from 10 liters of blood. We have used upwards of 150 liters of blood to prepare about 3.5 gm. of material, although as shown later some of the batches were completely lost.

The products thus prepared we refer to subsequently as Blood I, as only one copper hydroxide precipitation was used. In most cases the material was fairly easily removed from the tube after drying in alcohol but in no case was this product perfectly white. On heating in a capillary tube the substance often swelled to a very great extent before it actually decomposed. On continuing the heating the products decomposed from 187–193° but we have never obtained what could be regarded as a definite m.p. The phenomenon of swelling, which has been noticed by the Cambridge workers, appears to be due entirely to the compactness of the material. On the addition of alcohol the glutathione is precipitated as a gum which solidifies on standing, and the finally dried material thus weighs relatively more than might appear from its bulk.

The analysis of the Blood I products did not appear to us to fit satisfactorily the requirements of diglutaminylcystine (glutathione) and we reapplied the copper hydroxide precipitation in the hope of obtaining a purer product. We had prepared by evaporation from an alcoholic solution, followed by prolonged drying in an evacuated calcium chloride desiccator, about 0.85 gm. of glutathione, for the most part in the reduced form as shown by its specific rotation<sup>1</sup> of  $[\alpha]_D = -17^\circ$ . We took along with this 0.20 gm. of substance, which, until we had found its  $[\alpha]_D$  to be  $-83^\circ$ , we had regarded as oxidized material but which on retesting with sodium nitroprusside and ammonia and with the uric acid reagents we found still contained some reduced substance. The total substance, thus amounting to rather more than 1.0 gm. in a volume of 70 cc. of water, was reduced by adding moist copper hydroxide, passing hydrogen sulfide, and allowing to stand overnight in the hydrogen sulfide atmosphere. The copper sulfide was filtered off, hydrogen sulfide removed by aeration, and moist copper hydroxide added without delay to the solution

<sup>1</sup> See following paper, Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 147.

amounting to over 100 cc. The copper compound was centrifuged off and the supernatant liquid allowed to stand overnight in an ice chest. The copper compound was thoroughly washed with water, the copper removed as sulfide, and hydrogen sulfide removed from the solution with the afore mentioned precautions. After oxidation the solution was evaporated to about 3 cc. *in vacuo* and about 30 cc. of absolute alcohol were added. Unlike our crude preparations of glutathione the substance separated readily as a crystalline-like solid and not as an oil. On allowing to stand overnight the solid still remained disintegrated and only on the bottom of the tube was there a small surface from which the substance required to be removed with a glass rod. On microscopic examination the substance appeared to consist of finely divided crystalline material, yet we were not satisfied that we could discern any definite crystalline forms. In this fraction we obtained 0.6 gm. of a bulky pure white substance.

On the following morning the supernatant liquid in the ice chest contained well defined rosettes of crystals on the bottom and sides of the flask. These were composed of very friable and opaque material of a light bluish gray color. They were collected, washed, and treated as described for the other copper compound. We thus obtained 0.15 gm. of material similar to the first.

The material thus obtained showed only slight swelling when heated in a capillary tube. At 188–190° the substance decomposed to a very thick brown gum. This material is subsequently referred to as Blood II product.

*Special Difficulties in the Preparation of Glutathione from Blood.*—In our first attempt to prepare glutathione from blood we encountered no special difficulties other than a relatively great loss of nitroprusside-reacting material as noted by Hopkins at various stages in the preparation. So constant is this loss, even when the greatest care is taken to avoid it by the insurance of maximum precipitation and complete washing at each step, and by hastening the whole process as much as possible, that it might raise some doubt as to whether or not glutathione is the only substance in yeast or blood extracts which gives a positive nitroprusside reaction. Reduced glutathione is a relatively simple substance and it is very difficult to account for the small yields obtained as

compared to what we should expect from the nitroprusside test on the original extracts.

In the case of blood however we encountered the additional difficulty that the substance sought frequently decomposed with the evolution of hydrogen sulfide in the course of the fractionation. The first signs of decomposition always occurred before the first mercury sulfate precipitation in the course of removing barium sulfate from the acidified barium hydroxide filtrate. The smell of hydrogen sulfide was never perceptible immediately after acidification of the alkaline filtrate but generally appeared during the filtration. Once it did appear however it was unmistakable, and traces of lead salts in solution were precipitated as sulfide. The subsequent addition of the mercury sulfate reagent yielded a small dirty precipitate. Whenever such a decomposition occurred we discarded the whole batch.

We thus lost successively some five batches, or the product from about 50 liters of blood. For some time indeed the difficulty appeared to be insuperable and we tried many unsuccessful devices, such as precipitation with finely ground solid barium hydroxide and the omission of the barium hydroxide precipitation. We convinced ourselves however that the decomposition was not due to the barium hydroxide, and precipitation with barium hydroxide appears to be essential, as it requires repeated mercury precipitations to get rid even of the bulk of the substance precipitated by the barium.

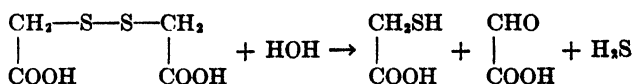
A similar difficulty was encountered at the phosphotungstic acid stage. We had accumulated the product from several batches, having kept them in an atmosphere of hydrogen sulfide, and after removal of the sulfide and adjustment of the solution to 0.5 normal acid with sulfuric acid we added a solution of 20 per cent phosphotungstic acid in 0.5 normal sulfuric acid. There was produced however, a precipitate of a rubbery consistency and hydrogen sulfide was also evolved. This precipitate, freed from phosphotungstic acid with barium hydroxide, gave only a faint nitroprusside reaction even with sodium cyanide as alkali and there was little glutathione in the phosphotungstic acid supernatant liquid. We were thus forced to abandon the phosphotungstic acid precipitation, and we have found it to be unnecessary if the first lead acetate precipitation is carefully carried out. We have

frequently tested small portions of our mercury filtrates but only a faint cloudiness was produced with the addition of the phosphotungstic acid. Of course sympectothion is the substance most likely to be precipitated at this stage.

At another stage we never failed to notice the production of hydrogen sulfide. As will be seen from the table of preparation a small amount of glutathione frequently remains unprecipitated by the lead and is subsequently carried down by mercury chloride along with sympectothion. When the mercury filtrate, freed from hydrogen sulfide, is precipitated in neutral solution with lead acetate a small precipitate consisting largely of the lead compound of glutathione is obtained. We have invariably found that hydrogen sulfide is evolved immediately sulfuric acid is added to this precipitate in excess.

We have no satisfactory hypothesis to offer to account for the formation of hydrogen sulfide from glutathione under those circumstances.

Contact with alkali does not explain the decomposition, as the glutathione of the sympectothion fraction has not been in contact with alkali. It is also difficult to see how free sulfur could be in any of the solutions. The decomposition may be of a kind similar to that noted by Dixon and Tunnicliffe (4) in the case of dithioglycollic acid. That is



*Properties of Glutathione Prepared from Blood.*—The analytical results of Blood I and Blood II products are shown in Table I of our paper on Glutathione,<sup>1</sup> alongside the corresponding values obtained from glutathione prepared from yeast and from liver.

#### DISCUSSION.

The bearing of the analytical results obtained from Blood I and Blood II products on the composition of glutathione is discussed in the succeeding paper. A comparison of the values with the corresponding values obtained from the yeast product and with the calculated values from the liver product makes it almost certain that the glutathione of the three products is identical.

*Amount Present in Normal Human Blood.*—Our observations on the probable amount of glutathione present in blood are yet only in the preliminary stages, but we have reason to believe that centrifuged corpuscles commonly contain 100 to 120 mg. of glutathione per 100 cc. Our values were obtained on the evidence of the nitroprusside reaction, using sodium cyanide as alkali and finally adding the sodium nitroprusside solution to the test solution cooled in a freezing mixture.

The test solution contained 5 cc. of 1:20 Folin-Wu corpuscle filtrate + 3 gm. of ammonium sulfate + 2.5 cc. of 30 per cent sodium cyanide.

The standard solution contained 0.25 mg. of glutathione in 5 cc. + 3 gm. of ammonium sulfate + 2.5 cc. of 30 per cent sodium cyanide.

The two test-tubes of equal bore were immersed in a freezing mixture for 5 minutes, then 0.2 cc. of saturated sodium nitroprusside solution was added to each at the same time. The tubes were then immediately inverted to mix, and the colors matched against a white background.

Thus two blood corpuscle filtrates showed that the test contained rather more color than the standard, and in two other filtrates the color was slightly less than in the standard.

We thus conclude that the corpuscles of two of the bloods contained slightly more than 100 mg. of glutathione per 100 cc. and two slightly less than 100 mg. of glutathione per 100 cc.

Washed sheep corpuscles showed about the same amount. Cat corpuscles showed 120 mg. of glutathione per 100 cc. and the gastrocnemius muscle from the same animal about 47 mg. per 100 gm. We thus conclude that whole blood contains about the same amount of glutathione as muscle, and corpuscles about twice as much as muscle.

*Bearing on Blood Analysis.*—In view of the great attention which has been paid to blood analysis in recent years it is a matter of some surprise that the presence of glutathione has not previously been observed. Its presence in corpuscles will now account for almost all of the amino nitrogen of protein-free corpuscle filtrates and for about 4 mg. of the "rest nitrogen" of those filtrates.

The demonstration of two new non-protein sulfur constituents of blood would seem to us to be of much interest from the point of

view of sulfur metabolism which has hitherto found its appropriate place under protein metabolism (see Cathcart (3)). Non-protein sulfur has indeed received little attention, and there appear to be few recorded analyses of the total non-protein sulfur of tissue extracts or even of blood filtrates, although such sulfur must occur in blood filtrates in significant amounts. Thus we should expect glutathione and sympectothion alone to account for 10 to 15 mg. of non-protein sulfur per 100 cc. of corpuscles. It has been shown that the sulfur metabolism of proteins runs very parallel with their nitrogen metabolism, and we should expect that new non-protein sulfur compounds will prove as interesting as non-protein nitrogenous substances have proved.

*On the Rotatory Power of Blood Filtrates.*—Holden's work on the subject of glutathione was undertaken "with the intention of investigating the optically active substances of blood other than glucose" and he concludes that the glutathione in blood is "the chief optically active constituent of de-proteinised blood other than glucose and as such is responsible for the effects recorded by Winter and Smith [(12)] and others and quoted as evidence in favour of the presence of an unstable modification of glucose." Holden further concludes that glutathione is in the corpuscles in the reduced form.

It is also our belief that glutathione is, at least for the most part, in the reduced form in blood. Holden, however, had not measured the specific rotation of reduced glutathione, otherwise he could not have come to such conclusions. For, as we have shown,<sup>1</sup> the specific rotatory power of reduced glutathione is likely to be a small positive value and the effect of the presence of glutathione in the Winter and Smith extracts is hardly predictable. If their treatment caused little oxidation of the glutathione, as might appear from their description, then the effect of glutathione on their rotations could be only very slight; yet if there was much oxidation it might be appreciable. Winter and Smith used whole blood so that glutathione is at least a factor to be considered.

Much attention has been given to the rotatory power of blood filtrates, or extracts, as contrasted with the reducing powers of such solutions towards reagents ordinarily used for the estimation of glucose. Notably, Lundsgaard and Holbøll (10) have postulated the presence in normal blood of a hitherto unrecognized form of glucose which they have called "new-glucose" on the



evidence solely of differences in the rotatory power and reducing power of blood dialysates. Like Winter and Smith they found that fresh blood dialysates showed less glucose by polarimetric measurement than by reduction methods, but on allowing them to stand for about 48 hours the rotatory power rose to correspond with the reducing power. Lundsgaard and Holbøll have considered the possible interference of creatinine and uric acid besides glucose in blood, and indeed they attribute a small percentage discrepancy in their calculations to the presence of those two substances.

Like Winter and Smith, Lundsgaard and Holbøll used whole blood, so that any non-protein optically active constituents of corpuscles would almost certainly be in their dialysates. We have shown that there is perhaps twenty times more glutathione in whole blood than uric acid, and the oxidation or reduction of this substance alone under varying conditions might simulate the slow mutarotation effects noticed by Winter and Smith and by Lundsgaard and Holbøll. Were these effects due however to glutathione alone we should expect the reverse effect, that is, too high a glucose value from rotation in fresh blood extracts and a lowered value on allowing them to stand, as the reduced glutathione normally present in fresh extracts readily oxidizes, and therefore the rotation would be lowered. It is impossible however to say definitely how the presence of glutathione would affect these findings without a complete revision of the work of Winter and Smith and of Lundsgaard and Holbøll with this point in view.

As we have shown elsewhere there is also present in blood another optically active compound which we have called sympectothion. This substance occurs in whole blood commonly in amounts varying from 10 to 15 mg. per 100 cc. and has a specific rotation of  $+115.0^\circ$ . If blood extracts are made without the destruction of this substance we should again expect to find a higher glucose value by rotation than by reduction with the glucose reagents in the freshly prepared filtrates. It is significant that the extracts of Winter and Smith rapidly decolorized potassium permanganate as does sympectothion also in solution.

Such remarks are perhaps sufficient to show that observations of the type recorded by Winter and Smith and by Lundsgaard and Holbøll cannot be satisfactorily interpreted without taking

into account these two substances which occur in large amounts in blood and which are both optically active. Indeed it appears quite accidental that the rotatory power of blood extracts should yield glucose values so closely in accord with those found by reducing methods, as the property of optical activity has no relation to copper-reducing power. The two properties happen to coexist in glucose but in neither glutathione nor sympectothion. And it is not improbable that the optical activity of both glutathione and sympectothion is often masked in blood extracts, especially if the glutathione has been largely oxidized in the course of extraction, in which case the negative rotation of oxidized glutathione will offset the positive rotation of sympectothion. And with the demonstration of these two optically active constituents of blood the whole tale is perhaps not yet told.

*Physiological Aspects.*—It is true that the demonstration of glutathione in blood corpuscles in amounts probably twice as great as in muscle does not invalidate present day conceptions of the metabolic significance of glutathione, which have been so ably suggested by the Cambridge school, any more than the presence of large amounts of glucose in blood invalidates the belief that the glucose is not metabolized there. The analogy is of course not strict but our conceptions of intracellular chemical processes are as yet so vague that the mere demonstration of a chemical constituent of a tissue extract can prove or disprove little or nothing. It is, however, evident that if glutathione is mainly or in large part responsible for oxidation and reduction in the organism, then it is more free to act in certain tissues than in others. For even accepting as holding true *in vivo* all the varied reactions which glutathione may induce *in vitro*, it must remain difficult to answer the question of why there should be approximately twice as much glutathione in blood corpuscles as in muscle.

#### SUMMARY AND CONCLUSIONS.

The isolation of glutathione from blood is attended by special difficulties and a method designed to overcome them has been outlined.

Crude and purified glutathione prepared by us from blood has the same composition as glutathione prepared from yeast and from liver but differs in composition from that prepared by Hopkins.

Evidence is given that glutathione is commonly present in human and animal corpuscles to the extent of 100 mg. per 100 cc.

There is more glutathione in mammalian corpuscles than in mammalian muscle.

The presence of glutathione in blood has been discussed from the point of view of blood analysis and from a physiological standpoint.

Some interpretations of differences between the rotatory and reducing powers of blood filtrates have been critically examined in view of the presence of glutathione and of sympectothion in blood filtrates.

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## GLUTATHIONE. A CRITICAL STUDY.

BY GEORGE HUNTER AND BLYTHE ALFRED EAGLES.

(*From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.*)

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On the evidence furnished by Hopkins (5) and Quastel, Stewart, and Tunnicliffe (6) glutathione in its reduced form is a simple dipeptide of glutamic acid and cysteine and oxidizes through its sulfydryl group to form the disulfide in a way analogous to the oxidation of cysteine to cystine. It was isolated by Hopkins from yeast, liver, and muscle.

In the preceding paper we have described the isolation from blood corpuscles of a substance which resembled glutathione, but the results of analysis of our product seemed to differ too much from the theoretical requirements to warrant the conclusion that it was identical with glutathione; for our problem at this stage was strictly that of proving or disproving the identity of the blood product with glutathione. We accordingly prepared glutathione from liver, the product from which was in some respects markedly dissimilar to the blood product, which dissimilarity we traced to the presence of cystine. We had perforce to prepare glutathione from yeast.

The examination of the yeast and blood products leaves little room for doubt that the two products are identical. But with that problem solved we are faced with much graver ones. In short the molecule of the reduced substance would appear to contain 3 nitrogen atoms, of which 2 exist as free amino groups in the intact substance and the third is exposed as an amino group after hydrolysis. The low sulfur content of our products would further indicate that glutathione is not a simple dipeptide of cysteine and glutamic acid but a combination of these with something additional. Our analytical findings would allow for the presence of serine in ester linkage, although we have no further evidence for the presence of this hydroxyamino acid.

We are well aware of the weight of evidence opposed to such a suggestion but our analytical findings would appear to necessitate it. We have, unfortunately, been unable to find in the literature the record of any other attempts to prepare and reexamine glutathione chemically for the reason perhaps that the evidence of the Cambridge workers would indicate that a further chemical investigation of glutathione was uncalled for. Indeed, we ourselves were only accidentally and reluctantly compelled into such an investigation. But our experience with the substance is perhaps worthy of record, for if our observations are seriously in error, then the preparation of glutathione is not so easy as Hopkins indicates.

#### EXPERIMENTAL.

*Preparation of Glutathione from Different Sources.*—The preparation of glutathione from liver was made substantially along the lines described by Hopkins. The preparation from blood we have described in detail in the preceding paper, while the preparation from yeast, probably because of the nature of our raw material, we had to modify in minor respects.

A 25 pound batch of Fleischmann's compressed yeast was suspended in 3 pound lots in 7 liters of water and, with constant stirring, brought to the boiling point and maintained there for 5 minutes. There was little coagulation of the protein with this treatment and separation by filtration or by centrifugation was quite impossible. The mixture was accordingly cooled by immersing the container in cold water and was treated in portions as described below with barium hydroxide.

We had at our disposal an 800 cc. centrifuge with two sets of tubes. Just sufficient of the cooled yeast suspension (about 1600 cc.) to fill two sets of centrifuge tubes was taken in a 3 liter flask and hot saturated barium hydroxide added until a good coagulum was obtained. This treatment precipitated most of the nucleic acid along with the protein. The alkaline fluid was immediately centrifuged in a period of 10 to 12 minutes, the supernatant liquids poured off and made just acid with 2 normal sulfuric acid. The remainder of the boiled and cooled fluid was treated similarly, so that the glutathione was in an alkaline medium only for a short time. No attempt was made to wash

the precipitates, as the time necessary to do so was not worth the questionable increase in yield. When a second 3 pound lot had been similarly treated the combined filtrates from the 6 pounds of yeast, amounting generally to about 12 liters, were precipitated with basic lead acetate made according to Goulard (4). The lead precipitate was allowed to settle, the supernatant liquid drained off, and the precipitate centrifuged and washed with water. It was collected in a large mortar, made to a paste with water, and 2 normal sulfuric acid added to slight excess.

The filtrate and washings were then precipitated with uranium acetate, made alkaline with barium hydroxide, and a clear colorless centrifugate obtained. The excess barium was removed with 2 normal sulfuric acid and the filtrate precipitated with the Hopkins and Cole mercury sulfate reagent. The precipitate was well washed with water and freed from mercury by hydrogen sulfide. The material was kept in presence of hydrogen sulfide at this stage until the remainder of the yeast was worked up.

Mercury sulfide was then filtered off from the several lots, the filtrates combined, hydrogen sulfide freed by aeration, and the fluid made approximately 0.5 normal with sulfuric acid. 20 per cent phosphotungstic acid in 0.5 normal sulfuric acid was added until precipitation ceased. The rather small precipitate was centrifuged off, the supernatant liquid freed from sulfuric acid by barium hydroxide, and the filtrate from the barium sulfate reprecipitated by acid mercury sulfate.

The mercury precipitate was treated as the previous one and the filtrate precipitated by freshly precipitated copper hydroxide in nearly neutral solution. The copper compound was centrifuged and washed until the supernatant liquid was almost free from sulfate.<sup>1</sup> It was suspended in water, the copper removed as sulfide and the hydrogen sulfide by a current of air washed successively in sodium hydroxide and sulfuric acid. To insure complete removal of hydrogen sulfide at this final stage a few drops of 2 normal sulfuric acid were added to the fluid and aeration continued for 2 to 3 hours.

<sup>1</sup> It is very difficult to get the last traces of sulfate freed from the copper precipitate, and as the copper compound of glutathione is appreciably soluble in water we have generally stopped with four washings, the water each time amounting to about twice the volume of the precipitate.

The solution was then adjusted to pH 7.5 and aerated until the glutathione was all in the oxidized form. The solution, exactly freed from barium and sulfuric acid, was then reduced *in vacuo* to a volume of about 5 cc. and about 30 cc. of absolute alcohol added. After standing for a day the friable product was filtered and washed with absolute alcohol in a small suction filter. It was finally dried to constant weight in an evacuated calcium chloride desiccator. The yield from 25 pounds of yeast was 3.16 gm. of an almost white powder. This amounts to about 28 mg. per 100 gm. of yeast and compares favorably with the best yields recorded by Hopkins.

The fractionation was followed in a rough quantitative way with the phosphotungstic acid and sodium carbonate test, and from this it seems improbable that the yield represents more than 10 per cent of the glutathione actually present.

All our products charred and decomposed between 187-193°. We have found no definite m.p. but similar decomposition points with products which later proved to be quite different in composition. In our experience m.p. determinations of glutathione are of little value.

For the purpose of this paper we report in Table I the main findings from five typical products. The product from yeast was obtained as we have described, and that under Liver I by the method of Hopkins. The product Blood I was prepared as we have described in the preceding paper, and those under Blood II and Liver II by a repetition of the copper precipitation on products obtained as for Blood I and Liver I.

#### *Hydrolysis.*

0.180 gm. of Blood I glutathione was hydrolyzed for 8 hours with 10 cc. of 7.6 normal hydrochloric acid. The volume was reduced to about 4 cc. and the fluid placed in a 15 cc. centrifuge tube. Dry hydrochloric acid gas was then passed through the fluid which was kept cold. Crystals appeared in less than 1 hour but the tube was stoppered and placed in an ice chest overnight. On microscopic examination on the following day two distinct types of crystal were present. One type, relatively few in number, resembled crystals of glutamic acid, which we had previously prepared by saturating a solution of glutamic acid with hydro-

chloric acid in a similar manner. But the microscopic field was filled mainly with rectangular spar-like crystals or coarse needles. A solution of pure cystine was saturated with hydrochloric acid under similar conditions and yielded very readily similar crystals apparently of cystine dihydrochloride.

The crystal mixture was accordingly centrifuged and washed twice with cold concentrated hydrochloric acid. The fluid was drained off as well as possible, a rubber stopper, through which was the end of a calcium chloride tube, was inserted, and the tube evacuated. When most of the hydrochloric acid had evaporated the tube was placed in warm water and the vacuum maintained for about an hour. The centrifuge tube was then detached and placed in an evacuated calcium chloride desiccator overnight. Next morning about 3 cc. of water were added to the dry crystal mixture which immediately dissolved, but the solution soon became cloudy with the separation of what appeared to be free cystine. Microscopic examination soon showed the typical hexagonal plates. After allowing the separation of cystine to proceed as far as possible, the tube was again centrifuged, the supernatant liquid reserved, and the crystals washed with water. They were redissolved in a small amount of hydrochloric acid, hydrochloric acid gas was again passed, and the typical spar-like crystals of cystine dihydrochloride readily separated in pure form. Free cystine was recovered in a way similar to that which we have described, except that the solution was finally made just alkaline with ammonium hydroxide and then just acid with acetic acid to get a complete precipitation. Further cystine was obtained from the first supernatant by adding barium hydroxide until the solution was left just faintly acid. The solution was allowed to stand for a day at this stage, after which it was centrifuged, the supernatant reserved, and the washed precipitate dissolved in a small volume of 2 normal hydrochloric acid. A trace of barium was removed with sulfuric acid, the fluid was again reduced to a small volume, and further cystine dihydrochloride, and subsequently cystine, recovered as we have described. The last reserved supernatant liquid was exactly freed from barium and sulfuric acid, reduced *in vacuo* to a small volume, and saturated with hydrochloric acid gas. The saturated solution was kept in an ice chest for 3 days, over which period crusts of glutamic acid



hydrochloride separated. This was washed with cold concentrated hydrochloric acid, taken up in a small volume of water, and reprecipitated as the hydrochloride. This was finally separated and dried as described for cystine dihydrochloride.

No attempt was made to isolate the cleavage products quantitatively. About 20 mg. of cystine and about the same quantity of glutamic acid hydrochloride were obtained in pure form from 180 mg. of the blood product.

0.200 gm. of Liver I glutathione was hydrolyzed in the same way and glutamic acid hydrochloride and cystine similarly isolated.

0.500 gm. of the yeast product was hydrolyzed with 20 cc. of acid and an attempt made to isolate cystine by repeated evaporation to dryness of the hydrolysate, taking up each time with water. The mass remained sticky even after eight such evaporations and too much hydrochloric acid remained in it to allow the dissociation of much cystine hydrochloride when dissolved in water. Some cystine however was filtered off at this stage and purified by precipitation as dihydrochloride. But the amount obtained did not indicate that this procedure was better than that previously adopted and to which we had recourse to isolate glutamic acid hydrochloride and further cystine from the yeast hydrolysate.

The hydrolyses described were performed strictly from a qualitative standpoint. They confirm the work of Hopkins in so far as they show that cystine and glutamic acid occur in glutathione hydrolysates. From his hydrolysates, however, Hopkins claims to have recovered some 90 per cent of the glutamic acid calculated as present in glutathione by concentration of the hydrolysate and saturating with hydrochloric acid as we have described. He also recovered about 60 per cent of the theoretical cystine but notably from a different hydrolysate obtained with sulfuric acid, and by precipitation as the mercury compound. We are quite unable to see how Hopkins, in the course of the precipitation of glutamic acid hydrochloride from the hydrochloric acid hydrolysate, prevented the precipitation of cystine dihydrochloride along with it. Of course, it is well known that glutamic acid is removed in this way from protein hydrolysates but we are not aware that it is usually done in presence of a large percentage of

cystine. But whether it succeeds or not under such circumstances, for it is quite possible that cystine dihydrochloride may be soluble and glutamic acid hydrochloride insoluble in a complex mixture of amino acids, the two substances cannot be separated in this way in simple mixtures. From our glutathione hydrolysates cystine dihydrochloride almost invariably appeared first, and always came down in largest quantity on allowing to stand; for the two types of crystals can readily be distinguished microscopically. In pure solution also, cystine dihydrochloride appears to be much more insoluble than glutamic acid hydrochloride in cold concentrated hydrochloric acid. Thus cystine dihydrochloride separates readily from 0.5 per cent solution even without immersion in ice water, and we have obtained the characteristic crystals in solutions of only half this strength. Glutamic acid hydrochloride we did not find to separate from 0.5 per cent solution even when allowed to stand saturated with hydrochloric acid gas in an ice chest for 24 hours.

Nor does the presence of glutamic acid hinder the precipitation of cystine under the conditions indicated. Thus from a synthetic mixture of glutamic acid and cystine in the proportions expected in a glutathione hydrolysate (118 mg. of glutamic acid and 96 mg. of cystine representing 200 mg. of glutathione in about 8 cc. of water) we obtained cystine dihydrochloride apparently in pure condition at first, but after standing for several days glutamic acid hydrochloride was also present.

We have confirmed these experiments many times as the insolubility of cystine dihydrochloride in concentrated hydrochloric acid does not seem to be generally recognized, although, interestingly enough, this property of cystine was used by Wollaston, its discoverer, as a means for its identification (see Garrod (3)). Further, the main direct evidence of Hopkins that glutathione contains only cystine and glutamic acid rests on his quantitative isolation of those amino acids, and in view of our findings that evidence would not appear to be acceptable.

*Estimation of Cystine in Hydrolysates.*—20 mg. of the different products were hydrolyzed for 8 hours with 5 cc. of 6 normal sulfuric acid. This was then diluted to 25 cc. Part was removed for the estimation of amino nitrogen as described in the protocols and cystine was estimated on the remainder. The method used

for the estimation is described elsewhere.<sup>2</sup> From Table I it is seen that 20 mg. of our preparations from yeast and from blood show the presence of about 7.6 mg. of cystine. The theoretical yield of cystine from 20 mg. of glutathione is 9.64 mg. Both of the liver preparations yield more than the theoretical amount of cystine on account of the presence of free cystine in the original preparations.

Sulfuric acid was used for this hydrolysis to minimize the destruction of cystine. It has been shown by Van Slyke (8) that more cystine is destroyed by hydrochloric acid hydrolysis than by sulfuric acid hydrolysis. We tested the action of the two acids on a mixture of 20 mg. of cystine and 24.6 mg. of glutamic acid; that is, in the proportion expected in a glutathione hydrolysate. One mixture was boiled for 6 hours with 5 cc. of 6 normal sulfuric acid and another for the same time with hydrochloric acid. In the case of the sulfuric acid there was a destruction of about 4 per cent of the cystine present as against a destruction of 20 per cent of the cystine in the case of hydrochloric acid.

*"Cystine Color Equivalent" of Glutathione from Different Sources.*

—It is shown in an accompanying paper<sup>2</sup> that cystine and glutathione can be estimated accurately in simple solutions, after reduction with sodium sulfite in sodium hydroxide solution, by measurement of the blue color produced on the addition of the Folin and Trimble (2) phosphotungstic acid reagent. It was thought desirable to determine the amounts of cystine and glutathione yielding the same color, and we have expressed this by recording the number of parts of cystine which give the same color by this method as is given by 10 parts of glutathione. This we have termed the "cystine color equivalent" of glutathione. It appears to us a convenient and accurate method of checking the purity of different glutathione preparations. The free cystine in the liver products is readily detected by it and the amounts of glutathione and cystine present in the preparations are calculable.

*Further Experimental Data.*—Further experimental data for the determination of total nitrogen, amino nitrogen, amino nitrogen after hydrolysis, and sulfur, with details of the measurement of specific rotation, are given in the protocols.

<sup>2</sup> Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 177.

TABLE I.  
*Analytical Findings from Glutathione Obtained from Blood, Yeast, and Liver.*  
 (For further explanation see text.)

Source.	Blood II.	Blood I.	Yeast.	Liver I.	Liver II.	Calculated for diglutaminylcystine.
Total nitrogen, <i>per cent.</i>	12.01 11.98	11.74	11.51 11.89	11.50	11.77	11.24
Amino nitrogen, <i>per cent.</i>	7.89	7.84 7.84	7.83 7.86	8.22	8.73	5.62
Amino nitrogen, <i>per cent.</i> After hydrolysis.	11.99 11.88	11.15 11.26	11.57 11.22	11.50 11.68	11.32 11.55	11.24
Sulfur, <i>per cent.</i>	10.27 9.97	10.00 9.34	9.97 8.85	13.59 (9.53)*	17.46 (10.21)*	12.85
$[\alpha]_D$ (1) 1 per cent in aqueous solution. (2) 4 " " " (3) 2 " " " 1 N hydrochloric acid. $[\alpha]_{HCl}$ 1 per cent in aqueous solution.	-93.9°   -111.0°	-93.5°   	-93.5° -89.9° -81.5°	-142°	-175°   	$[\alpha]_{HCl}$ 1. Approximately 3.5 per cent in aqueous solution = -98.3°. 2. Approximately 2.0 per cent in 10 per cent hydrochloric acid = -89.2°. (Stewart and Tunnicliffe).
Cystine equivalent of 10.0 mg.	4.64	4.53	4.52	5.93	7.00	
Mg. of cystine in hydrolysate from 20 mg.	7.6	7.5	7.7	9.97	11.30	9.64

\* Calculated for glutathione in mixture.

## DISCUSSION.

An examination of our tabulated data for the different products at once reveals a close similarity in the products from yeast and blood, and many discrepancies in the product from liver. The liver product indeed may profitably be left for discussion elsewhere, as it is a mixture of glutathione and free cystine.

We shall first discuss the data in the order tabulated in their relation to those required by diglutaminylcystine (glutathione).

*Total Nitrogen.*—Little emphasis can perhaps be placed on the discrepancy between our findings and the requirement of glutathione. Nevertheless the average of the six values given for the yeast and blood products is 11.82 per cent as compared with the theoretical value of 11.24 per cent. The difference is certainly not due to experimental error and our products proved to be free from ammonia when tested with Nessler's reagent.

*Amino Nitrogen.*—Our average amino nitrogen value of 7.85 per cent is 2.23 per cent higher than the theoretical of 5.62 per cent. But despite the discrepancy it is doubtful if much weight can be attached to it, for the reason that free cystine gives abnormal values by the Van Slyke (9) method. As Hopkins (5) found a considerably higher value for the amino nitrogen of free cystine than did Van Slyke we thought it desirable to have our own figures. The values found are shown.

*Per Cent Amino Nitrogen of Cystine.*

Van Slyke.	Hopkins.	Writers.	Theoretical.
12.52	14.5	12.76	11.67
12.68		12.87	

Taking our own values, which are slightly higher than Van Slyke's, cystine yields about 10 per cent too much amino nitrogen, and assuming the same error in the estimation of amino nitrogen in glutathione the expected yield would then be about 6.2 per cent. But as Hopkins notes, a correction for the amino nitrogen of glutathione based on the values found for cystine would not necessarily be satisfactory. Indeed it may be quite incorrect to add as much as 10 per cent to the theoretical figure, and on the other hand it is possible that a much greater correction must be applied. Taken by itself the high amino nitrogen of our products is perhaps of little significance but the fact that it is two-thirds

of the total nitrogen is certainly strongly suggestive of the presence of two free amino groups in the glutathione molecule.

*Amino Nitrogen after Hydrolysis.*—As shown in the protocols our technique for the preparation of the test solutions involved perhaps an unnecessary amount of manipulation and consequently a probable loss of material. Our values for amino nitrogen after hydrolysis are thus somewhat lower than might be expected when free cystine is present, but they are all sufficiently close to the total nitrogen to leave no doubt that all the nitrogen in the hydrolyzed product is in the amino form. And further, it is clear that, unless the correction error for the amino nitrogen of the original glutathione is unexpectedly high, the amino nitrogen of glutathione is not doubled by hydrolysis but increased by half, indicating that the glutathione molecule contains not 2 but 3 nitrogen atoms.

*Sulfur.*—We are inclined to regard the sulfur values of our preparations as of more significance than any other single factor. The average of the six values given for the yeast and blood products is 9.73 per cent, which is less than the theoretical requirement of 12.85 per cent by 3.12 per cent. It can further be readily shown by calculation that glutathione from liver contains approximately the same amount of sulfur as do the yeast and blood products. By calculation from the cystine color equivalent of the crude liver product a value of 9.53 per cent of sulfur is obtained for the glutathione in the mixture, and by a similar calculation the glutathione in the pure liver product contains 10.21 per cent of sulfur. These calculations are more fully explained in the following paper.<sup>3</sup>

It is probable that our sulfur values err in being too high rather than too low. After fusion our solutions were precipitated with a considerable excess of barium chloride in lukewarm solution and allowed to stand overnight. We have since found that under these conditions a little barium chloride tends to attach itself to the barium sulfate and is very difficult to remove by washing with cold water as was our practice. Indeed it is only on some such hypothesis that we can explain the high results obtained from using small amounts of cystine. Thus when using

<sup>3</sup>Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 172.

about 25 mg. of cystine we obtained the values 27.79, 27.90, and 27.90 per cent of sulfur instead of 26.69 per cent required by calculation. Better values were obtained when larger quantities of cystine were employed. Thus with 100 mg. of cystine we obtained 26.83 per cent of sulfur and in another control, using 61.18 mg. of cystine and 74.32 mg. of glutamic acid, we obtained the value of 27.3 per cent of sulfur for cystine or 13.17 per cent calculated for the mixture representing glutathione.

A tripeptide containing cystine, glutamic acid, and serine would contain 12.46 per cent of nitrogen and 9.49 per cent of sulfur. The total nitrogen in the yeast and blood products we find to be approximately 12.0 per cent and the sulfur 9.7 per cent. For the reasons just stated the sulfur content is more probably about 9.5 per cent, so that if the nitrogen is slightly low the values for sulfur and nitrogen taken together are strongly suggestive of the presence of serine in glutathione along with cystine and glutamic acid. And if our supposition is correct that glutathione contains one amino acid other than glutamic acid and cystine, one of the peptide linkages will not be of the ordinary type but probably of an ester type, so that 2 of the 3 nitrogen atoms will exist as free amino groups.

*Specific Rotation.*—The specific rotations,  $[\alpha]_D$ , obtained from 1 per cent aqueous solutions of the yeast and blood products are in substantial agreement, and, taking into consideration the greater angle given by the mercury line, our values are about 10 per cent higher than that previously recorded by Stewart and Tunnicliffe (7). There is evidence that the specific rotation may be affected by dilution as Andrews (1) has shown to be the case with cystine, but it is undoubtedly much less variable than in the case of cystine. When our readings were taken from a 4 per cent solution our value for the specific rotation of oxidized glutathione is still about  $5^\circ$  higher than that given by Stewart and Tunnicliffe. When taken in 1.0 normal hydrochloric acid the fall in specific rotation is of about the same magnitude as that found by Stewart and Tunnicliffe with 10 per cent hydrochloric acid solution.

The very high rotation of the liver product first betrayed to us the presence of cystine.

It might be expected that the presence of a small amount of

cystine in the yeast and blood products would account for the specific rotation being higher than that recorded by Stewart and Tunnicliffe. Were free cystine present, however, we should expect to get a greater percentage of sulfur, as we actually find in the liver product; but moreover we describe elsewhere a test which precludes the possibility of free cystine raising the rotation of the yeast and blood products.

The fact that reduced glutathione has only a very small negative rotation, or possibly a positive rotation, has apparently escaped the notice of the Cambridge workers. Our attention was first called to this fact when we found the specific rotation of one of our products to be unexpectedly low ( $-83^\circ$ ). On testing this product with phosphotungstic acid and sodium carbonate we found that a blue color was produced, and a further test with sodium nitroprusside and ammonia showed the presence of reduced glutathione in our dried product. From a too economical use of testing material during the aeration the presence of the reduced substance had escaped notice.

As shown in the protocols a batch of the reduced blood product showed a specific rotation of  $-17^\circ$ . This sample had been evaporated *in vacuo* without oxidation by aeration but had suffered some oxidation in the course of evaporation.

Another sample of 1.5 gm. was reduced with hydrogen sulfide in presence of copper hydroxide and the sulfide-free filtrate read in the polarimeter. The specific rotation for this batch was found to be  $-4.74^\circ$ .

It is likely, even with this simple process, that a small amount of the glutathione was oxidized, so we may safely conclude that reduced glutathione has probably a small positive rotation, as Andrews has recently shown to be the case with cysteine. This finding is significant when the rotatory power of tissue extracts is being examined. We have discussed this more fully in the preceding paper.

*Cystine Color Equivalent.*—With respect to the color-yielding power of the different products towards phosphotungstic acid, the yeast and blood glutathiones are again in substantial agreement. Blood II glutathione we regard as the best we have prepared and this color test shows it to be so. Using a cystine standard for the estimation of glutathione we can thus calculate the glutathione equivalent.



It is impossible to say with certainty what amount of color glutaminylcystine would produce relative to that obtained from cystine. We should expect the SH group of glutathione to have the same reducing power towards phosphotungstic acid as the SH group of cystine, in which case the colors produced by equal weights of substance would be inversely proportional to their molecular weights. We should thus expect 10 mg. of glutathione to produce the same amount of color as 4.82 mg. of cystine. Our finding that 10 mg. of glutathione are equivalent to 4.64 mg. of cystine is evidence in favor of the presently accepted molecular weight of glutathione rather than in favor of the evidence from amino nitrogen and sulfur values. It is unfortunate, however, that little weight can be attached to this finding.

*Estimation of Cystine in Hydrolysates.*—The cystine values obtained from the hydrolysates from the yeast and blood products again agree very well. About 80 per cent of the cystine of the theoretical dipeptide is accounted for and yet it is very difficult to believe that there should be a destruction of 20 per cent of the cystine on hydrolysis with sulfuric acid, especially when our cystine values agree very well with what we should expect from the total sulfur.

#### SUMMARY AND CONCLUSIONS.

Glutathione has been prepared from yeast, blood, and liver, and the products from the different sources appear to be chemically identical when allowance is made for the presence of free cystine in the liver product. Our preparations, however, are at variance, especially as to content of total nitrogen, amino nitrogen, and sulfur with the theoretical requirements of di-glutaminylcystine. The specific rotation of our products is higher than that previously reported, and the specific rotation of the reduced product has been shown to have a very low negative, if not a positive, value.

Our results suggest that reduced glutathione is not a simple dipeptide of cystine and glutamic acid, although those two amino acids are present.

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We further wish to express our thanks to Professor V. J. Harding for help in many ways and to Professor J. W. Bain of the Department of Chemical Engineering for assistance with the Carius method.

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*Addendum.*—A number of papers relative to the occurrence of glutathione in blood have come to our notice since the present papers were submitted for publication. Harding and Cary (11) from a study of cystine metabolism were led to suspect the presence of glutathione in blood but dis-

continued their attempts to isolate it on the appearance of the paper by Holden. They showed however by the use of the Folin-Looney reagents for cystine that there were probably appreciable quantities of the substance in blood. An unsuccessful attempt to isolate the labile sulfur-containing substance of blood has recently been recorded by Campbell and Geiling (12). They found it possible to account for the total labile sulfur of the serum by the labile sulfur of its protein constituents.

Meanwhile several records of the glutathione content of blood have also appeared. Thus Thompson and Voegtlin (13), and also Uyei (14) have found in blood by the iodometric method of Tunnicliffe values of like magnitude to those we have here reported on the basis of the nitroprusside test.

Of greatest interest to us however has been the appearance of papers by Bergmann and Stather (15) and by Brand and Sandberg (16) on the subject of labile sulfur. Abel and Geiling (17) appear to have been the first to differentiate "labile" sulfurs when they showed that the sulfur of insulin is less firmly bound than is the sulfur of cystine. When the paper by Abel and Geiling appeared we tested glutathione by their sodium carbonate method and came to the conclusion that the sulfur of glutathione was apparently as unstable as that of insulin, which, through the kindness of Dr. D. A. Scott of the Connaught laboratories, we had at the same time the opportunity of testing. After we found that glutathione had no hypoglycemic effect on intravenous administration to rabbits the hypothesis of Abel concerning the parallelism between labile sulfur and hypoglycemic effect appeared to us to fail as a general statement although it may still hold good with respect to insulin. The significance of the degree of lability of the sulfur is however especially emphasized in the two papers referred to above, and the work of Bergmann and Stather is very suggestive in the light of our experience in the preparation of glutathione from blood. In the behavior of dialanyl-L-cystine dianhydride in slightly alkaline solution we have a type of decomposition, with evolution of hydrogen sulfide, which appears to parallel closely the behavior of glutathione under the conditions we have described. It is significant that the sulfur of dialanycystine, the true analogue of diglutaminylcystine is much less labile than that of the dialanycystine dianhydride. As an explanation of the greater instability of the dianhydride Bergmann and Stather suggest that the formation of the dioxypiperazine ring weakens the bond holding the sulfur. And, although this is perhaps not an adequate explanation, as has been pointed out by Brand and Sandberg, it nevertheless leaves open the possibility that some similar transformation occurs in the glutathione molecule prior to the decomposition which we have noted. Such a suggestion is not unsupported, from the fact that Bergmann and Stather form the dianhydride of dialanycystine through the intermediary dimethyl ester which is apparently very easily transformed by cold ammonia to the unstable dianhydride, in view of our suggestion that glutathione contains serine in ester linkage. It would thus seem not improbable, when glutathione decomposes in the spontaneous manner we have described, that the serine, or other amino constituent, under certain conditions splits off with the formation of a residue of

the nature of dialanylecystine dianhydride. For, our experience would indicate that under certain conditions in the course of the preparation of glutathione, the substance is even more completely and more rapidly decomposed than is indicated by its behavior on boiling with sodium carbonate or standing with sodium hydroxide and lead.

In this regard also the dithiopiperazine described by Johnson and Burnham (18) should perhaps be recalled, as this substance on hydrolysis yields glycine and hydrogen sulfide, as also the more recent synthesis by Gatewood and Johnson (19) of an acylthion-amino acid which has also an unstable sulfur.

With regard to the note by Hopkins,<sup>4</sup> which has been submitted to us by the Editors, we naturally consider that the findings of Brand and Sandberg, with respect to the sulfur of glutathione, confirm our own. Nor have we any reasons to believe that any desulfurized substance was carried to our end-products. When decomposition did occur, it was so complete and so rapid that such batches were entirely discarded. In our first experiences of decomposition we did attempt further fractionation but precipitates in such cases were almost negligible in the glutathione fractions. And for this reason it would appear that the desulfurized product is not carried to the final stage as Hopkins suggests. By our modified method of preparation there was no hydrogen sulfide perceived at any stage.

As far as the use of barium hydroxide is concerned, our method was the same as that of Hopkins with the exception that we centrifuged the mixture instead of filtering it so that the time of contact with the barium hydroxide would be as short as possible.

#### PROTOCOLS.

##### *Hydrolytic Products of Glutathione.*

##### *Blood II.*

1. Glutamic acid hydrochloride, rhomboidal plates, m.p. 205°. Glutamic acid hydrochloride made from glutamic acid of known purity when heated at about the same rate, m.p. 208°. Amino nitrogen, Van Slyke micro apparatus, 5.76 mg. gave 0.78 cc. N at 23° and 754.5 mm. = 7.54 per cent. Calculated, 7.63 per cent.
2. Cystine, hexagonal plates, typical solubility in acid and alkali. Amino nitrogen, 4.68 mg. gave 1.06 cc. N at 23.5° and 756 mm. = 12.61 per cent. Calculated + 10 per cent = 12.84 per cent.

##### *Liver I.*

1. Glutamic acid hydrochloride, m.p. 208°. Amino nitrogen, 7 mg. gave 0.93 cc. N at 23° and 755 mm. = 7.40 per cent.
2. Cystine. Amino nitrogen, 4.68 mg. gave 1.05 cc. N at 23.5° and 756 mm. = 12.48 per cent.

##### *Yeast.*

1. Glutamic acid hydrochloride, m.p. 209°. Amino nitrogen, 7.92 mg. gave 1.14 cc. N at 22.5° and 755.5 mm. = 7.37 per cent.

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<sup>4</sup> Hopkins, F. G., *J. Biol. Chem.*, 1927, lxxii, 185.

2. Cystine. Amino nitrogen, 4.45 mg. gave 1.00 cc. N at 23.5° and 756 mm. = 12.5 per cent.

*Total Nitrogen.*

Total nitrogen was estimated by the usual Kjeldahl method with sulfuric acid and a small piece of copper. Each determination was checked by a blank containing the same amounts of sulfuric acid and sodium hydroxide and the titration values given are those obtained with the blank subtracted. Calculated value 11.24 per cent.

*Blood II.*

0.0500 gm. required 21.45 cc. 0.02 N NaOH. N = 12.01 per cent.

0.0500 " " 21.40 " 0.02 " " " = 11.98 " "

*Blood I.*

0.100 gm. required 8.39 cc. 0.1 N NaOH. N = 11.74 per cent.

*Yeast.*

0.1356 gm. required 11.15 cc. 0.1 N NaOH. N = 11.51 per cent.

0.1180 " " 10.03 " 0.1 " " " = 11.89 " "

*Liver I.*

Data lost. N = 11.50 per cent.

*Liver II.*

0.100 gm. required 8.41 cc. 0.1 N NaOH. N = 11.77 per cent.

*Amino Nitrogen.*

Amino nitrogen was determined by the micro apparatus of Van Slyke. The blank obtained from the sodium nitrite and acetic acid has been subtracted from the volumes given. Calculated value 5.62 per cent.

*Blood II.*

10 mg. gave 1.42 cc. N at 18.5° and 737.5 mm. = 7.89 per cent.

*Blood I.*

10 mg. gave 1.37 cc. N at 17° and 753.5 mm. = 7.84 per cent.

10 " " 1.37 " " " 17° " 753.5 " = 7.84 " "

*Yeast.*

10 mg. gave 1.365 cc. N at 17.5° and 759.5 mm. = 7.86 per cent.

15 " " 2.04 " " " 17.5° " 759.5 " = 7.83 " "

*Liver I.*

8.0 mg. gave 1.17 cc. N at 18.5° and 746 mm. = 8.22 per cent.

*Liver II.*

10 mg. gave 1.52 cc. N at 16° and 753 mm. = 8.73 per cent.

*Amino Nitrogen after Hydrolysis.*—Van Slyke micro apparatus. Blanks subtracted. 20 mg. hydrolyzed for 8 hours with 5 cc. 6 N H<sub>2</sub>SO<sub>4</sub>, then diluted to 25 cc. 15 cc. taken, neutralized with 7.0 cc. 2.5 N NaOH, and evaporated to about 4 cc.; then made to 10 cc. Calculated value 11.24 per cent.

*Blood II.*

4 cc. gave 1.00 cc. N at 15° and 751 mm. = 11.99 per cent.

4 " " 0.99 " " " 15° " 751 " = 11.88 " "

*Blood I.*

4 cc. gave 0.92 cc. N at 15° and 755 mm. = 11.15 per cent.  
 2.87 " " 0.67 " " " 15° " 755 " = 11.26 " "

*Yeast.*

4 cc. gave 1.005 cc. N at 23.5° and 752 mm. = 11.57 per cent.  
 3 " " 0.73 " " " 23.5° " 752 " = 11.22 " "

*Liver I.*

4 cc. gave 0.970 cc. N at 15° and 755 mm. = 11.68 per cent..  
 3 " " 0.715 " " " 15° " 755 " = 11.50 " "

*Liver II.*

4 cc. gave 0.94 cc. N at 15° and 755 mm. = 11.32 per cent.  
 3 " " 0.72 " " " 15° " 755 " = 11.55 " "

*Sulfur (Method of Warunis (10)).*

Substance heated in nickel crucible in water oven for 1 hour with 3.0 gm.  $\text{Na}_2\text{O}_2$  and 5.5 gm. KOH, then with small flame until completely fused. Fused mass dissolved in water, transferred to beaker, acidified with concentrated HCl saturated with bromine. Bromine boiled off with stirring and filtered solution precipitated with 5 cc. of 10 per cent barium chloride. Calculated value is 12.85 per cent.

*Blood II.*

0.050 gm. gave 0.0374 gm.  $\text{BaSO}_4$  = 10.27 per cent.  
 0.0521 " " 0.0379 " " = 9.97 " "

*Blood I.*

0.100 gm. gave 0.0728 gm.  $\text{BaSO}_4$  = 10.00 per cent.  
 0.0573 " " 0.0390 " " = 9.34 " "

*Yeast.*

0.1754 gm. gave 0.1275 gm.  $\text{BaSO}_4$  = 9.97 per cent (Carius method).  
 0.1030 " " 0.0664 " " = 8.85 " "

*Liver I.*

0.0992 gm. gave 0.0982 gm.  $\text{BaSO}_4$  = 13.59 per cent.

*Liver II.*

0.0878 gm. gave 0.1124 gm.  $\text{BaSO}_4$  = 17.46 per cent.

*Specific Rotation.*

The specific rotation was determined in all cases in a 2 dm. tube in a Schmidt and Haensch polarimeter. The reading given is the average generally of twelve readings and sometimes, when concordance was good, of only six readings. With the exception noted the specific rotations are all  $[\alpha]_D$ .

*Blood II.*

0.200 gm. in 20 cc. =  $\frac{-1.878^\circ \times 100}{2 \times 1} = -93.9^\circ$ .

Same solution  $[\alpha]_{\text{Hgl}}^{16^\circ} = \frac{-2.22^\circ \times 100}{2 \times 1} = -111.0^\circ$ .

*Blood I.*

$$0.15 \text{ gm. to 15 cc. with water} = \frac{-1.87^\circ \times 100}{2 \times 1} = -93.5^\circ.$$

*Yeast.*

$$0.2000 \text{ gm. to 20 cc. with water} = \frac{-1.87^\circ \times 100}{2 \times 1} = -93.5^\circ.$$

$$0.600 \text{ " " 15 " " " " } = \frac{-7.19 \times 100}{2 \times 4} = -89.9^\circ.$$

$$2 \text{ per cent solution of glutathione in 1 N HCl} = \frac{-3.26^\circ \times 100}{2 \times 2} = -81.5^\circ.$$

*Liver I.*

$$1.5 \text{ gm. to 50 cc. with water} = \frac{-8.52^\circ \times 100}{2 \times 3} = -142^\circ.$$

*Liver II.*

$$0.200 \text{ gm. in 20 cc.} = \frac{-3.5^\circ \times 100}{2 \times 1} = -175^\circ.$$

$$10 \text{ cc. of above solution} + 10 \text{ cc. 20 per cent HCl (6.18 N)} = \frac{-1.10^\circ \times 100}{2 \times 0.5} = -110^\circ.$$

*Partially Reduced Glutathione.*

$$0.2000 \text{ gm. in 20 cc.} = \frac{-1.66^\circ \times 100}{2 \times 1} = -83^\circ.$$

$$0.8435 \text{ " " 25 " " } = \frac{-1.15^\circ \times 100}{2 \times 3.386} = -17^\circ.$$

$$1.5 \text{ gm. oxidized glutathione in presence of Cu(OH)}_2. \text{ H}_2\text{S passed over-} \\ \text{night. Filtrate free from CuS and H}_2\text{S; volume 285 cc.} = \frac{-0.050^\circ \times 100}{2 \times 0.53} = \\ -4.72^\circ.$$

## ON THE PRESENCE OF CYSTINE IN LIVER.

BY GEORGE HUNTER AND BLYTHE ALFRED EAGLES.

*(From the Department of Pathological Chemistry, University of Toronto;  
Toronto, Canada.)*

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The evidence from the literature for the presence of cystine or cysteine in normal animal tissues is scanty. Before the discovery of glutathione by Hopkins (7) it was well known that protein-free extracts of most mammalian tissues give a positive nitroprusside test which was attributed to the presence of a free sulfydryl group. Arnold (2), by a series of qualitative tests in tissue extracts parallel with the same tests in cysteine, came to the conclusion that cysteine was present in the tissues. Arnold's evidence was almost as complete as it could have been without the actual isolation of cysteine or cystine. But since Hopkins' discovery, the qualitative tests used by Arnold are of no significance from our point of view as they can all be attributed to the presence of glutathione. Indeed no evidence in the earlier literature, short of the actual isolation of cystine, would be of significance. An isolation is recorded by Cloetta (3) from ox kidney and it seems not improbable from Cloetta's description of his method that he did isolate cystine which was not a hydrolytic product. It is, of course, uncertain whether that cystine was a cleavage product of glutathione, although it is not improbable that cystine as such may be present in the kidney. It is also recorded that Drechsel (4) isolated cystine from the liver of the dolphin and that he showed it to be present in the liver of the horse. We have not had access to Drechsel's paper on the latter subject.

It is thus plain that the evidence for the presence of cysteine or cystine in normal healthy tissues cannot be dismissed although it must be regarded as insufficient.

We were led to the discovery of the presence of cystine in



normal pig liver from the finding of an extraordinarily high rotatory power for what we regarded as glutathione prepared from that source. This high rotatory power we show to be due to cystine admixed with the glutathione, and we furnish some evidence that in liver tissue the substance is in the reduced form.

#### EXPERIMENTAL.

*Preparation.*—Glutathione was prepared from pig liver according to the method of Hopkins. The material, amounting in one case to 7.65 kilos, was brought to the laboratory, minced, and heating started with an equal weight of water and an equal weight of 0.1 normal sulfuric acid within 30 minutes of the time of slaughter. The mixture was heated to 83° at which temperature there was a good coagulation. The material was then treated in the usual way.

The final yield of almost white dried material was 2.67 gm. or about 35 mg. per 100 gm. of liver. This material is subsequently referred to as Liver I glutathione. It decomposed at about 190°.

We had previously obtained a crystalline copper compound of glutathione from the supernatant liquid after the final copper hydroxide precipitation in the case of the material obtained from blood. At this stage we did not know that the liver compound contained cystine and an attempt was made to get a purified product by repetition of the copper precipitation on some of the material prepared above after reduction. Before filtration the mixture containing the copper precipitate was heated to about 60° and filtered at this temperature, with the object of getting a greater yield of crystals in the supernatant liquid. After standing for a day the supernatant liquid did not yield crystals and glutathione was recovered from the precipitate in the usual way. The yield from 1.5 gm. of the original substance was only 0.45 gm., so that there was relatively a large loss of material by this treatment. The material was perfectly white and decomposed on heating at about 204°. It is subsequently referred to as Liver II.

*Analysis of the Product.*—The analytical findings in the two products Liver I and Liver II are tabulated alongside the corresponding values found for glutathione prepared from blood and

from yeast in Table I in the preceding paper. For the two products we give the values found for total nitrogen, amino nitrogen before and after hydrolysis, sulfur, and specific rotation. We have also measured the cystine color equivalent of the products and the amount of cystine in their hydrolysates.<sup>1</sup>

*Isolation of Cystine from the Liver Product.*—When we had obtained all the experimental evidence sought from product Liver II, the remainder of the substance, amounting to 60 mg., was dissolved in about 2 cc. of water and a drop of 2 normal ammonium hydroxide added to make the solution faintly alkaline. This was followed by a drop of 2 normal acetic acid to make distinctly acid and the solution was allowed to stand for several days in a corked tube. A small amount of precipitate appeared after several hours and after 2 days there was a precipitate amounting to about 10 mg., which on microscopic examination exhibited the hexagonal crystals characteristic of cystine. The crystals were washed several times with water in a small centrifuge tube to free from glutathione. They proved from qualitative tests to have all the properties of cystine.

*Colorimetric Test for Distinguishing Cystine from Glutathione.*—It has been noted by Sullivan (9) that cystine gives a color with  $\beta$ -naphthoquinone-4-sodium sulfonate in presence of sodium sulfite and sodium cyanide, and that glutathione does not give a color. Such a test was very valuable from our point of view, and as Sullivan gave no details of concentration of the reagents or technique of the test we were forced to work these out for ourselves.<sup>2</sup> We have used the following solutions.

1.  *$\beta$ -Naphthoquinone-4-sodium sulfonate* in 0.5 per cent aqueous solution, freshly prepared. We prepared the substance from 1-amino-2-naphthol-4-sulfonic acid according to the method of Folin (5).

<sup>1</sup>Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 147.

<sup>2</sup> Since the present paper was submitted for publication an exhaustive report has been published by Sullivan (11) on his new test for cysteine and cystine. Sullivan uses sodium hydroxide as alkali and allows the color to develop in the cold. To destroy any colors given by interfering substances, a large number of which he has tested, he employs sodium hyposulfite. As used by Sullivan the test appears to be somewhat more delicate than that described here.

2. *Sodium sulfite*, 20 gm. to 100 cc. with water, freshly prepared.

3. *Sodium cyanide*, 5 gm. to 100 cc. with water.

We have found the test to work satisfactorily for cysteine and cystine when performed as follows:

*Cysteine*.—The test solution at a volume of 1 cc. or less, is made faintly alkaline with sodium carbonate. To this are added 2 cc. of sodium sulfite and 0.2 cc. of the  $\beta$ -naphthoquinone solution. When heated in a boiling water bath for 2 to 3 minutes a purplish red color appears when cysteine is present to the extent of more than 0.1 mg.

*Cystine*.—To give the test cystine must be reduced to cysteine by means of sodium cyanide, although a color does develop but very slowly in presence of alkali and sodium sulfite alone.

To the test solution, as for cysteine, are added 1 cc. of sodium cyanide, 2.0 cc. of sodium sulfite, and 0.2 cc. of  $\beta$ -naphthoquinone. When heated as for cysteine a similar color appears.

We have tested both reduced and oxidized glutathione and find that no color is developed under the conditions outlined for cysteine or cystine.

Although the  $\beta$ -naphthoquinone reagent is that used by Folin for the estimation of amino acids, the color obtained with the test described here is not chemically the same as that obtained from amino acids. The color obtained by Folin is stable in acetic acid solution, whereas the color obtained in this test for cystine disappears on acidification with acetic acid. Further, the test is not given by amino acids other than cystine, so that we have here a very valuable means of testing for the presence of cysteine and cystine in tissues in the presence of glutathione.

All our glutathione preparations from liver have shown cystine to be present on the evidence of this test. Our preparations from blood and yeast on the contrary showed no trace of color by this test.

#### DISCUSSION.

The analytical findings reported in Table I of the preceding paper<sup>1</sup> provide in themselves almost conclusive evidence for the presence of cystine in products Liver I and Liver II. As cystine contains approximately the same amount of nitrogen as gluta-

thione, there is no evidence in the total nitrogen percentage for the presence of cystine, but the amino nitrogen values are even higher than those found for the blood and yeast products. The main evidence lies in the amount of sulfur present, in the high rotation, and in the high color values of the products before and after hydrolysis.

The amount of cystine in the products is calculable from several of the data. As there appears to us to be some doubt about the percentage of sulfur in pure glutathione it is perhaps not desirable to base such calculations on the sulfur values. In the case of the specific rotation a difficulty also arises in that the specific rotation of cystine, as has been shown by Andrews (1), varies over a large angle, about  $70^\circ$ , when measured under different conditions. The highest rotation for cystine recorded by Andrews is  $-268^\circ$ , which is much greater than is generally recorded in the literature. In our case, however, the rotations were measured in aqueous solution or rather in glutathione solution, the glutathione providing a solvent for the cystine. It is thus impossible to say what the specific rotation of cystine should be under such circumstances, although as it is in solution in a weak acid we should expect the rotation to be at least at the high end of Andrews' scale.

We regard the cystine color equivalents of the products as the most satisfactory data for the calculation of the amount of cystine present in the preparations. Assuming that 10 mg. of pure glutathione give as much color as 4.64 mg. of cystine by our method of estimation<sup>3</sup> then the amount of cystine and glutathione respectively is readily calculable for a product of which 10 mg. is equivalent to 5.93 mg. of cystine.

Thus if  $C$  = number of mg. of cystine in 10 mg. of mixture  
 and  $G$  = " " " " glutathione " 10 " " "  
 then for product Liver I

$C + G = 10$

and  $C + 0.464 G = 5.93$

Whence  $G = 7.59$  mg. and  $C = 2.41$  mg.

Or, the product Liver I contains 24.1 per cent of cystine and 75.9 per cent of glutathione.

<sup>3</sup>Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 177.

In the same way the product Liver II can be shown to contain 44 per cent of cystine and 56 per cent of glutathione.

The specific rotation of the admixed cystine can be calculated from the rotations found if we assume that  $[\alpha]_D$  for pure glutathione is  $-94^\circ$ .

It may be seen from the protocols in the preceding paper<sup>1</sup> that 1.5 gm. of Liver I product in 50 cc. of solution were used to measure the rotation. The angle found for this 3 per cent concentration in a 2 dm. tube was  $-8.52^\circ$  giving a specific rotation of  $-142^\circ$ .

It is shown from the color values that 24.1 per cent of the Liver I product is cystine. The amount of cystine in 100 cc. of the 3 per cent solution is thus 0.723 gm. and  $3 - 0.723 = 2.277$  gm. of glutathione.

If  $B$  be the angle given by the glutathione in the solution we have

$$\frac{B \times 100}{2 \times 2.277} = -94^\circ; \text{ when } B = -4.28^\circ.$$

But the rotation actually found for this solution was  $-8.52^\circ$ . So that the angle given by the cystine present is

$$-8.52^\circ + 4.28^\circ = -4.24^\circ.$$

Whence for cystine

$$[\alpha]_D = \frac{-4.24^\circ \times 100}{20 \times 0.723} = -293^\circ.$$

By a similar calculation for Liver II product, it can be shown that  $[\alpha]_D$  for the cystine present is  $-278^\circ$ .

It is not improbable that the specific rotation of cystine measured under these conditions is in the vicinity of what we have just found. At least it is very improbable, from a consideration of this calculated rotation, that the substance admixed with the liver products can be other than normal *l*-cystine.

Further evidence that our products from liver are simple mixtures of glutathione and cystine are obtainable from the percentages of sulfur found in the substance.

Thus 100 gm. of Liver I product contain, according to the color values, 24.1 gm. of cystine. 100 gm. of cystine contain 26.69 gm. of sulfur, so that 24.1 gm. of cystine contain 6.41 gm. of

sulfur. But it is found that 100 gm. of Liver I product contain 13.59 gm. of sulfur, so that 75.9 gm. of glutathione contain  $13.59 - 6.41 = 7.18$  gm. of sulfur, or the percentage of sulfur in the glutathione of the mixture is 9.53.

In the same way, as Liver II product contains 44 per cent of cystine and 17.46 per cent of sulfur, it can be shown that the percentage of sulfur of the glutathione in the mixture is 10.21.

A comparison of the sulfur values thus deduced for glutathione from liver shows a surprisingly close agreement with those found for glutathione prepared from blood and yeast. Such considerations indicate not only that free cystine is present in the liver product, but that glutathione of liver is identical with glutathione obtained from blood or yeast.

The final consideration from the analytical data is the amount of cystine found in the hydrolysates from the liver products. It will be seen from Table I of the preceding paper<sup>1</sup> that 20 mg. of Liver I product yield 9.97 mg. of cystine, which is slightly more than is theoretically required by Hopkins' formula for glutathione, and about 20 per cent more than the amount of cystine which we should expect to be present in the substance. In the hydrolysate of Liver II product the amount of cystine is even greater, as we should expect to find from a consideration of our other results.

Positive evidence for the presence of cystine in glutathione preparations from liver is thus obtainable from a consideration of the analytical data of our products, especially from the high specific rotation, the high sulfur content, the high color value, and the high cystine content of the hydrolysates; and the analytical findings are amply confirmed by the  $\beta$ -naphthoquinone test and by the isolation of typical cystine crystals from one of our preparations.

Our proof indeed may appear to have been unnecessarily urged. Yet we have thought it worth while to utilize all the evidence, not only because there are so many pitfalls in this type of research, but because the presence of free cystine or cysteine in tissues has not hitherto been convincingly shown.

That the cystine in our liver preparations is not an artifact is rendered almost certain by several considerations. In the first place, our products from blood and yeast contained no cys-

tine although they were prepared by the same general method. The product from a different batch of liver than that described above also showed the presence of cystine as indicated by a cystine color equivalent of approximately 6.7 and a positive  $\beta$ -naphthoquinone test. In the second place, the tungstic acid filtrate from dog liver was found to give a distinctly positive test with  $\beta$ -naphthoquinone and sodium cyanide, whereas a similar blood filtrate showed a negative test. This indicates that if free cysteine is present in blood it is there in very small amount.

That the substance in liver is cysteine rather than cystine is indicated by the fact that tungstic acid filtrates give a positive  $\beta$ -naphthoquinone test without the use of sodium cyanide. It is probable however that there is an equilibrium between the reduced and oxidized forms.

*Physiological Significance.*—We have not systematically investigated the distribution of cystine in the tissues but preliminary observations would suggest that liver is especially rich in this amino acid, which is autooxidizable in presence of tissue in a manner similar to glutathione. The presence of large amounts of cystine in liver, however, is suggestive from another point of view; that is, as the source of taurine for the formation of taurocholic acid. It is generally believed that cholic acid is the limiting factor in the formation of taurocholic acid (see Garrod (6), Whipple (10), Lewis (8)), the presence of a reserve of cystine being assumed. In an Eck fistula liver it is stated by Whipple that there is a subnormal production of bile acids. Whipple believes that the bile acids are formed only by the activity of the hepatic epithelium. Our findings would appear to indicate that there is in the liver a sufficient supply of cystine for the formation of taurocholic acid.

The significance of the liver in cystine metabolism is also indicated by experiments of Blum described by Lewis. Blum found that cystinuria was produced by injection of cystine into the peripheral circulation but was not observed when the cystine was injected into the mesenteric venous circulation. It is stated by Lewis that Blum's work "suggested that this difference might be due to a specific influence of the liver on cystine catabolism, possibly through retention of the cystine in the liver tissue for

the synthesis of taurine." It would thus appear that one of the errors in cystinuria lies in the incapacity of the liver to handle the cystine normally in reserve there.

#### SUMMARY.

It has been shown that glutathione preparations made from pig liver contain free cystine. The presence of cystine in the products accounts for their high specific rotation, high sulfur content, high color values when tested with phosphotungstic acid by a method described elsewhere,<sup>3</sup> and high cystine content of the hydrolysates.

Pure cystine has been isolated from liver.

Cystine has been shown to be present by a color test with  $\beta$ -naphthoquinone both in the glutathione preparations and in protein-free liver extracts.

It is suggested that the free cystine in liver may be of importance in the formation of taurocholic acid and that one of the manifestations of cystinuria may lie in a hepatic failure to metabolize the free cystine of liver.

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## THE COLORIMETRIC ESTIMATION OF CYSTINE AND GLUTATHIONE.

By GEORGE HUNTER AND BLYTHE ALFRED EAGLES.

*(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)*

(Received for publication, June 23, 1926.)

The method described in the following paper for the estimation of cystine and glutathione is the outcome of an attempt to measure accurately the amount of cystine in glutathione hydrolysates and, secondly, to have a reliable means of estimating the total glutathione, oxidized or reduced, in suitably prepared solutions. Tunnicliffe (3) has already described an iodometric method for the estimation of reduced glutathione in tissue extracts and has produced some evidence to show that the glutathione in those extracts is all in the reduced form. Preliminary observations have led us to believe that this is not quite so, but at any rate it seems desirable to have a method other than iodine titration, not only because the latter estimates only reduced glutathione but because, in blood filtrates at least, one substance other than reduced glutathione absorbs iodine. We refer to sympectothion.

The application of our method to the estimation of glutathione in tissue extracts is at present under investigation. The method here described is for the estimation of the pure substance in aqueous solution. By the same method cystine should be measurable in protein hydrolysates, if the assumption of Folin and Looney is correct, that there are no other substances in protein hydrolysates yielding a color by their method.

Our investigations naturally started by a trial of the method for the estimation of cystine as described by Folin and Looney (1). Their method no doubt gives approximately accurate results if the standard and test solutions are developed simultaneously, as they advise, and provided also that the readings are not far apart. The authors state that the color developed

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by cystine by their method is stable for at least 1 hour, and they make no mention of the use of lithium sulfate to prevent clouding. Using an aqueous solution containing 70 gm. of anhydrous sodium carbonate in 500 cc. and following their method in other details, we invariably found a clouding within a few minutes, and if this was postponed by the addition of lithium sulfate, the color of the diluted solution was quite unstable. The description of Folin and Looney is rendered somewhat vague by the use of the term "saturated sodium carbonate" but trial of both the anhydrous and decahydrate saturated solutions gave similar results. With one-quarter the concentration of sodium carbonate we did, however, obtain solutions which apparently did not fade even on standing overnight and of course did not cloud. The colors developed from such solutions were roughly proportional when the test and standard solutions read near each other but showed great disproportionality when relatively small amounts of cystine were used. The disproportionality was due to the blue color given by the reagents.

Thus, when using sodium carbonate we were faced with two difficulties. When small amounts were used the reagents themselves gave a blue color, and when large enough amounts were used to eliminate this blank the solutions clouded when lithium sulfate was not used, and when it was successfully used to prevent clouding the colors were too unstable to be of use.

The chief difficulty of the method described by Folin and Looney lies in the use of sodium carbonate as alkali. This is easily seen from a consideration of the constituents of the solution.

Sulfurous acid itself reduces the special phosphotungstic acid reagent to give a blue color. The phosphotungstic acid reagent has an acidity of approximately 3.0 normal acid which thus neutralizes a considerable portion of the alkali used. If this alkali be sodium carbonate the addition of the phosphotungstic acid liberates carbon dioxide which necessarily remains as carbonic acid in the presence of the alkali. And in the presence of sodium sulfite the free carbonic acid liberates a certain proportion of sulfurous acid which of course reduces the phosphotungstate with the production of the blue color. It is indeed the presence of free carbonic acid and consequently of free sulfurous acid which is responsible for the reagent blank even in presence of a large excess of sodium carbonate.

The difficulty is readily overcome by the use of sodium hydroxide instead of sodium carbonate as alkali. With the test solutions described later it can easily be shown that the presence of carbon dioxide gives a blue color in the blank solution. Thus if two tubes be taken containing in solution sodium sulfite and sodium hydroxide, and carbon dioxide bubbled through one for 1 minute, the addition of the phosphotungstic acid will be followed by the appearance of a blue color in the solution through which the carbon dioxide was passed, but by no color in the other solution.

Ammonium hydroxide is a suitable alkali so far as the question of free sulfurous acid is concerned, but solutions in which it is used cloud readily even in presence of large amounts of lithium sulfate.

As our results show, however, sodium hydroxide in the concentration recommended would appear to be a perfectly suitable alkali.

#### EXPERIMENTAL.

##### *Solutions Required.*

*Cystine*.—1.0 normal sulfuric acid containing 1 mg. of cystine in 1 cc.

*Glutathione*.—Aqueous solutions containing 1 mg. in 1 cc.

*Sodium Hydroxide*.—1.0 normal solution free from carbon dioxide.

*Phosphotungstic Acid*.—According to Folin and Trimble (2).

*Lithium Sulfate*.—20 gm. to 100 cc. with water.

*Sodium Sulfite*.—20 gm. to 100 cc. with water, freshly prepared.

##### *Method.*

*Cystine*.—The method is readily seen from Table I, the constituents being added in the order named and with a specific interval of time when noted. The solutions were mixed in a small wide test-tube, 1 inch in diameter and 4 inches long. We have always made a practice of neutralizing the test solutions if these are acid before the addition of 2.0 cc. of 1.0 normal sodium hydroxide, so that reduction takes place in all cases in approximately the same concentration of alkali. This is rendered necessary especially when relatively large amounts of acid are added

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with the substance, as the 2.0 cc. of 1.0 normal sodium hydroxide are just slightly more than prevent the development of a blue color in the blank.

The full amount of color is developed in 5 minutes and the solutions can be matched immediately after the final dilution.

TABLE I.

*Reagents and Readings for the Colorimetric Estimation of Cystine.*

Range 0.05 to 0.40 mg. of cystine in a volume of 20 cc. Standard 0.2 mg. of cystine in 20 cc.

Sequence and amounts of test solutions and reagents.

Cystine solution.	1.0 N NaOH.	Water (to 1 cc.).	Li <sub>2</sub> SO <sub>4</sub>	1.0 N* NaOH.	Na <sub>2</sub> SO <sub>4</sub>		Phosphotungstic acid.*		Water (to 20 cc.).	Reading of test.	Reading of standard.
cc.	cc.	cc.	cc.	cc.	cc.	Interval 1 min.	cc.	Interval 5 min.	cc.	mm.	mm.
0.05	0.05	0.90	0.50	2.00	2.00		0.50		14.0	15.0	3.6
0.10	0.10	0.80	0.50	2.00	2.00		0.50		14.0	15.0	7.5
0.15	0.15	0.70	0.50	2.00	2.00		0.50		14.0	15.0	11.2
0.20	0.20	0.60	0.50	2.00	2.00		0.50		14.0	15.0	15.0
0.25	0.25	0.50	0.50	2.00	2.00		0.50		14.0	15.0	18.7
0.30	0.30	0.40	0.50	2.00	2.00		0.50		14.0	15.0	22.4
0.35	0.35	0.30	0.50	2.00	2.00		0.50		14.0	15.0	26.2
0.40	0.40	0.20	0.50	2.00	2.00		0.50		14.0	15.0	30.1

\* This phosphotungstic acid reagent, originally designed for other purposes, was nearly 3.0 normal acid on account of the fact that lithium carbonate was not incorporated as recommended by Folin and Trimble. When lithium carbonate is added according to these workers the acidity of the reagent, determined by titration against sodium hydroxide, using phenolphthalein as indicator, is about 1.7 normal. Consequently, correspondingly less sodium hydroxide should be used in the tests. With the complete Folin and Trimble reagent the lithium sulfate may be dispensed with, the amount of 1.0 normal sodium hydroxide used should be 1.4 cc., and water added so that the volume in which the color is developed is exactly 6.0 cc.

We have found no fading within 15 minutes and the colors are probably stable for a longer period.

The standard used to obtain the readings given in Table I contained 0.20 mg. of cystine in a volume of 20 cc. The test solution plunger of the Duboscq colorimeter in each case was set

at 15.0 mm. and the plunger in the cup containing the standard was moved to match. The readings obtained are thus direct readings, the greater the amount of substance present the greater being the reading.

*Glutathione.*—The method for the estimation of glutathione is exactly the same as that used for cystine. Our solutions of

TABLE II.

*Color Values of Glutathione Obtained from Different Sources.*

Range 0.1 to 0.8 mg. of substance in a volume of 20 cc. Standard 0.2 mg. of cystine in 20 cc.

Blood II.			Blood I.			Yeast.			Liver I.		
Amount.	Reading of test.	Reading of standard.	Amount.	Reading of test.	Reading of standard.	Amount.	Reading of test.	Reading of standard.	Amount.	Reading of test.	Reading of standard.
mg.	mm.	mm.	mg.	mm.	mm.	mg.	mm.	mm.	mg.	mm.	mm.
0.1	15.0	3.5	0.1	15.0	3.5	0.1	15.0	3.4	0.1	15.0	4.4
0.2	15.0	7.0	0.2	15.0	6.8	0.2	15.0	6.8	0.2	15.0	8.8
0.3	15.0	10.5	0.3	15.0	10.1	0.3	15.0	10.2	0.3	15.0	13.3
0.4	15.0	14.0	0.4	15.0	13.7	0.4	15.0	13.7	0.4	15.0	17.8
0.5	15.0	17.3	0.5	15.0	16.9	0.5	15.0	16.8	0.5	15.0	21.9
0.6	15.0	21.2	0.6	15.0	20.4	0.6	15.0	20.2	0.6	15.0	26.1
0.7	15.0	24.3	0.7	15.0	23.6	0.7	15.0	23.3	0.7	15.0	30.5
0.8	15.0	27.4	0.8	15.0	26.7	0.8	15.0	27.3	0.8	15.0	35.2
Average reading for 0.1 mg. of substance 3.485			Average reading for 0.1 mg. of substance 3.40			Average reading for 0.1 mg. of substance 3.39			Average reading of 0.1 mg. of substance 4.395		
Cystine equivalent* = 4.64			Cystine equivalent = 4.53			Cystine equivalent = 4.52			Cystine equivalent = 5.93		

\* This number of parts of cystine gives the same amount of color as 10 parts of the glutathione preparation.

glutathione were aqueous so that no preliminary neutralization was necessary, the amount of alkali used for this purpose in the cystine tests being replaced by the corresponding amount of water. The solutions were matched against a standard containing 0.20 mg. of cystine in a volume of 20 cc.

The properties of the different samples of glutathione examined

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in Table II are discussed elsewhere. The product Blood II was specially purified, those under Blood I and yeast being crude products. The product from liver contains cystine as we prove elsewhere.

*Estimation of Cystine in Yeast Glutathione Hydrolysate.*—20 mg. of yeast glutathione were hydrolyzed for 8 hours with 5 cc. of 6 normal sulfuric acid. The cooled and slightly diluted solution was nearly neutralized with sodium hydroxide and made to a volume of 25 cc. Three portions were taken from this solution and cystine was measured as we have described. The readings are shown in Table III. The amount of cystine in the 25 cc. of hydrolysate is readily calculable from  $\frac{23}{15} \times 0.2 \times 25 = 7.7$  mg. That is, 20 mg. of hydrolyzed glutathione yield 7.7 mg. of cystine.

TABLE III.

*Cystine in Hydrolysate from 20 Mg. of Yeast Glutathione in Final Volume of 25 Cc. Standard 0.2 Mg. of Cystine in Volume of 20 Cc.*

Amount of hydrolysate.	Reading of test.	Reading of standard.
cc.	mm.	mm.
0.3	15.0	7.0
0.6	15.0	14.2
0.9	15.0	20.3
1.0	15.0	23.0 (calculated).

### DISCUSSION.

It may be seen from Tables I, II, and III that the colors obtained are strictly proportional to the amounts of cystine or glutathione present over the ranges 0.05 to 0.4 mg. and 0.1 to 0.8 mg. respectively. It is probable that proportionality is maintained within even wider limits. We regard such a proportionality as the best criterion of the value of a quantitative colorimetric method.

We have chosen cystine as a convenient standard for the estimation of glutathione by the method described for the reason that cystine is easily obtainable in very pure form. Our purified glutathione Blood II we regard as pure glutathione, yet there must remain some doubt on this point until certain difficulties,

described elsewhere,<sup>1</sup> have been cleared up. For this reason it is the more desirable to use cystine as a standard for its estimation so that a common dependable basis may be established for the comparison of glutathione prepared by different workers.

Under Blood II, Table II, we show that the cystine color equivalent is 4.64 for the purest glutathione we have been able to prepare. So that when cystine is used as a standard the values obtained should be multiplied by the factor  $\frac{10}{4.64} = 2.16$  to give the corresponding amount of glutathione.

The method is not yet applicable to the estimation of glutathione in tissue extracts for the reason that certain other substances present give a blue color with the reagents. At least three of these are cysteine, uric acid, and sympectothion. Cysteine and sympectothion will, of course, interfere with the iodine method already described by Tunnicliffe for the estimation of reduced glutathione.

#### SUMMARY.

A colorimetric method is described for the accurate estimation of cystine and glutathione in simple solutions. It is shown that 4.64 parts of pure cystine yield the same amount of color as 10 parts of glutathione.

The amount of cystine in hydrolyzed glutathione has also been measured.

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## ON THE ISOLATION OF GLUTATHIONE.

By FREDERICK GOWLAND HOPKINS.

(From the Sir William Dunn Institute of Biochemistry, University of Cambridge, Cambridge, England.)

(Received for publication, November 27, 1926.)

Hunter and Eagles have applied to blood, mammalian liver, and yeast the method (slightly modified) originally used for the isolation of glutathione, and in each case obtained a product which, though yielding glutamic acid and cystine on hydrolysis, differed in several respects from the substance described by me under the above name. In particular their products contained considerably less sulfur.

At the wish of the Editors of this *Journal* and by the courtesy of the authors I was allowed to read the papers which describe their results<sup>1</sup> in advance of their publication.

Brand and Sandberg<sup>2</sup> have also recently referred to a preparation of glutathione made by them from ox blood which contained only 8.6 per cent of sulfur instead of the 12.87 required by theory. These authors assume however that their product was impure, whereas Hunter and Eagles base upon their results the suggestion that glutathione is not a dipeptide, but a more complex substance, possibly a tripeptide, containing, it is true, glutamic acid and cysteine, but in association with some other amino acid. I venture to think from the available evidence that the former authors interpret their experience more correctly than the latter.

I may first remark that many preparations of glutathione have been made in this laboratory during the last few years which have in all respects agreed with the product as originally described. Since the synthesis by Stewart and Tunncliffe<sup>3</sup> I have never seen occasion to doubt the accepted constitution of glutathione, and I

<sup>1</sup> Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 133, 147.

<sup>2</sup> Brand, E., and Sandberg, M., *J. Biol. Chem.*, 1926, lxx, 381.

<sup>3</sup> Stewart, C. P., and Tunncliffe, H. E., *Biochem. J.*, 1925, xix, 207.

feel that Hunter and Eagles in their discussion have too much ignored the careful work of the above mentioned authors. Products made here from yeast have agreed in the minutest particulars with that obtained by Stewart and Tunnicliffe by their second synthetic method.

Incidentally I may be allowed to say that I resent somewhat the implication that I did not succeed in quantitatively separating glutamic acid from cystine in the study of the hydrolytic products as described in my first paper. In the particular case referred to by Hunter and Eagles the glutamic acid separated, which constituted 90 per cent of that required for the dipeptide constitution, was, as my notes show, entirely free from sulfur.

It is nevertheless clear that Hunter and Eagles are fully justified in publishing their results, especially as the analytical data yielded by the three products handled by them are not without concordance. Their experience however may only show that my original description of the method, whereby the dipeptide or its disulfide has always been isolated in this laboratory, is not precise enough for others to succeed when working from the description alone. One point bearing on this possibility may be at once mentioned. My assistant, E. J. Morgan, long ago observed that the sulfur in glutathione is much more labile than that in cystine or cysteine. In the interesting paper by Brand and Sandberg already mentioned this fact has been demonstrated and brought into line with the behavior, *e.g.*, of the analogous substance dialanylecystine. The observations of the last mentioned authors represent an extension of those of Bergmann and Stather who had previously demonstrated the lability of the sulfur in the dioxopiperazine anhydride of dialanylecystine.

The sulfur in pure glutathione, alike in its thiol and disulfide form, would seem to be at least as labile as that in the compounds mentioned. The following qualitative observations are sufficient to show this.

10 mg. of pure glutathione were dissolved in 5 cc. of each of the solutions to be mentioned and 2 drops of 5 per cent neutral lead acetate added in each case. With  $N/10$  sodium carbonate immersed in a boiling water bath marked blackening could be observed after 20 minutes. In the case of cold saturated barium hydroxide (0.38  $N$ ) the mixture when allowed to stand at room

temperature (14.5°C.) became yellow in 1 minute, brown in 5 minutes, and deep black in 15 minutes. With barium hydroxide saturated at 38°C. (*circa* normal) there was instantaneous blackening on the addition of the lead salt. Cystine when dissolved in the last mentioned solution and kept in a bath at 38°C. showed no trace of color within 5 hours. The above observations were made with the disulfide; others with the thiol form gave results of the same order.

The presence of lead undoubtedly catalyzes the desulfuration. If, however, the glutathione be allowed to stand in the alkaline solution without lead, and the progress of the removal of sulfur tested from time to time by noting the evolution of  $H_2S$  on acidification, the marked instability is still evident. Thus at room temperature in 0.38 normal barium hydroxide there was marked evolution of  $H_2S$  upon adding acid after 6 minutes, and after 15 minutes the gas was freely evolved.

Now at two stages in the course of the isolation of glutathione as described by me the materials are treated with hot barium hydroxide. If there be any delay at these stages there is grave danger of loss of sulfur from the dipeptide. A preliminary investigation seems to show that if this has occurred the desulfurized product (whatever its nature), accompanies the glutathione to the final stages of the process.

I think these circumstances may have contributed to the low sulfur content of the products obtained by Hunter and Eagles, but there were doubtless others.

In any case, although I have myself no doubt as to the nature of glutathione, the appearance of Hunter and Eagles' papers makes it desirable that I should if possible give greater precision to the account of its isolation. This I hope to do in the near future.



# INTERRELATIONSHIPS BETWEEN THE CHEMICAL COMPOSITION OF THE BLOOD AND THE LYMPH OF THE DOG.\*

BY ROSSLEENE M. ARNOLD AND LAFAYETTE B. MENDEL.

*(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)*

(Received for publication, December 4, 1926.)

The present investigation is concerned with the interchanges that take place between the blood, the lymph, and the tissues. While extensive studies of the chemical composition of the blood under a large diversity of conditions are available, there is a comparative paucity of information respecting the lymph and the tissue fluids. Most of the investigations have been complicated by the profound anesthesia and other features incident to the methods of experimentation employed. Some of these by themselves have an effect upon the blood that may obscure the fundamental phenomena under consideration. An attempt has consequently been made to avert as far as possible such undesirable complications. It was found that by the use of the anesthetic amytal (isoamyl-ethyl-barbituric acid), reputed to be without effect upon the content of sugar in the blood (Page (1)), the composition of the blood and the lymph of dogs remained almost constant for periods of 6 to 18 hours. For the present purpose therefore animals under such anesthesia were considered to be essentially "normal." After a determination of the relationships of some of the constituents of the blood serum and the lymph under such "normal" conditions, experimental interferences with physiological functions were initiated. Under the "abnormal" conditions which followed, alterations comparable to the changes which occurred in the concentrations of the con-

\* The data reported in this paper are taken from the dissertation presented by Rossleene M. Arnold in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1926.

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stituents of the blood were regularly apparent in the lymph. Fairly constant ratios were thus found to exist at all times between the constituents of the two fluids, showing that diffusible substances pass easily and rapidly between the blood and the lymph.

### *Methods.*

The experiments were conducted upon medium sized dogs. Preliminary studies of the extent to which such factors as size, sex, and nutritive condition of the animals might influence the composition of the blood showed that for none of the blood constituents was the range of variation sufficiently large to render the use of animals of special type or nutritive condition imperative for the success of comparable experiments (Table I). The diet consisted, as a rule, of dog biscuits until the day preceding the operation, when food was withheld but water was supplied in abundance. The animals were anesthetized early in the morning. During the day they were kept warm with an electric pad. Lymph was collected continuously in successive approximately equal portions. Samples of blood were removed at intervals of 1 or more hours corresponding with the beginning and the end of the periods during which the successive portions of lymph were taken. The experiments were continued as long as colorless lymph and serum could be obtained.

For the *anesthesia* amytal was given preference. It was injected intraperitoneally in doses sufficiently large to maintain the animals in operative condition for periods of 6 to 18 hours. When morphine and ether were used for anesthesia, morphine sulfate was injected subcutaneously 1 hour before the administration of ether by insufflation. Alcohol, used for anesthesia, was given by stomach tube in dilute solution. With it an almost perfect operative condition was produced, but the blood was so altered that it became impossible to separate adequate samples of serum for analysis.

Samples of *blood* were drawn either from one of the external jugular veins, from the heart, or from the femoral vein. Serum was prepared as follows: the blood was drawn into mineral oil in a syringe, was delivered under oil into a centrifuge tube which was closed with a rubber stopper, and centrifuged at once for

TABLE  
Composition of Whole Blood (W) and Blood Serum (S) of Normal Dogs.

Dog.	Weight.	Food* (water <i>ad libitum</i> ).	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.	Protein N.
	kg.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
Female.	6	Standard food.†	404 (S)	10.3 (S)	3.5 (S)	118 (S)	26.1 (S)	
"	9	"	328 (W)	10.5 "	3.6 "	144 (W)	37.6 (W)	
"	10	"	353 "	11.4 "	4.5 "	125 "	35.2 "	
"	10	Dog biscuit.	386 (S)	10.4 "	4.2 "			
"	15	"	388 "	10.6 "				
"	15	" " meat, milk, bone ash.	334 (W)	10.8 "	4.3 "	120 (W)	28.2 (S) 39.5 (W)	
Male.	12.5	Dog biscuit.		10.9 "	4.5 "		32.1 (S)	0.92 (S)
"	14.2	"		10.8 "	4.9 "		27.0 "	0.90 "
"	15.5	"	393 (S)	10.6 "	3.6 "	117 (S)	30.0 "	0.91 "
"	19.5	"	403 "	10.4 "	3.8 "	127 "	28.2 "	0.90 "
"	32.0	"	408 "		3.7 "	129 "	29.1 "	

\* Prior to the removal of blood, the dogs went without food for about 24 hours.

† Cowgill "standard diet" made up of isolated foodstuffs: casein, sucrose, and lard, together with a salt mixture and vitamin-bearing products.



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15 to 20 minutes. The serum was thus separated from the cells without exposure to the air. The serum was siphoned off and measured out in samples. Analysis of the samples was begun immediately.

The *lymph* flowed from a small glass cannula inserted into the thoracic duct, and was collected under a layer of mineral oil in a funnel closed at the stem end with a rubber tube and a pinch-clamp. The oil not only prevented the evaporation of the lymph but also delayed its clotting. The lymph usually flowed fast enough, 10 or more cc. each hour, so that it did not clot in the cannula. From the clotted lymph the clear fluid was pressed out with glass rods, discharged from the funnel, and filtered. Often this filtrate clotted again and again; whereupon the process was repeated until a limpid fluid remained. Samples were measured out and analysis of the samples was begun immediately.

Serum and lymph were analyzed for calcium, phosphorus, chloride, protein nitrogen, non-protein nitrogen, and sugar. Hemoglobin determinations also were usually made on the blood. Selected methods of analysis were adapted to the volumes of materials accessible. *Calcium* was determined according to Tisdall's method modified by Clark and Collip (2). For *phosphorus*, Briggs' modification of the Bell and Doisy method described by Myers (3) was used. *Non-protein nitrogen* determinations were made on a trichloroacetic acid filtrate. Folin's method for digestion and nesslerization, described by Myers (3), was followed with the modification that the digestion mixture was leached with water and filtered quantitatively into volumetric flasks before nesslerization. *Protein nitrogen* was determined by the Kjeldahl method on the protein precipitated by trichloroacetic acid. *Sugar* was estimated according to the method of Lewis and Benedict modified by Myers and Bailey and described by Myers (3). For *chloride*, Myers' method of 1920 (4) was applied to some of the picric acid filtrate remaining from the sugar determinations. The method of Cohen and Smith (5) was used for *hemoglobin*. The value for the first sample of blood drawn was designated as 100 and used as a standard for subsequent determinations. The values for hemoglobin are thus relative and, in so far as hemoglobin is a dependable index, indicate changes in concentration of the blood.

In the tables records are given of eight experiments which are *typical* of a large number of investigations carried out to show the relationships between the chemical composition of the blood and the lymph of the dog.

#### EXPERIMENTAL RESULTS.

##### *Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia.*

The data presented in Table II are typical of three experiments. The figures show that the composition of the serum and the lymph remained almost constant throughout the period of observation after the injection of amytal.<sup>1</sup> The values for the concentrations of hemoglobin were identical in all the samples of blood taken. The content of total solids in the two fluids did not change. No alterations occurred in the concentrations of protein nitrogen. These facts indicate that neither the serum nor the lymph exhibited changes in concentration. The values for calcium and phosphorus remained constant, the concentrations of the other constituents showing at most only slight variations throughout the 10 hours of observation.

The constant composition of the serum and the lymph thus justifies the inference that dogs under amytal anesthesia are essentially "normal." In these animals, at any rate, the uniformity of the quantitative relationship between the constituents of the two fluids is clearly demonstrated. A summary of the data presented in Table II permits ready comparison of the relationships between the concentrations of some of the constituents of the serum and the lymph.

<sup>1</sup> Amytal solution in N/2 NaOH, prepared according to the directions of Eli Lilly and Company, was injected in sufficiently large amounts (usually at least double the dose recommended) to produce complete anesthesia. Since this manuscript was prepared it has been demonstrated that amytal has little effect on the basal metabolism of the dog; and the sugar content of the blood remains unchanged for many hours during anesthesia with the drug. Cf. Deuel, H. J., Jr., Chambers, W. H., and Milhorat, A. T., *J. Biol. Chem.*, 1926, lxi, 249.

TABLE II.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia.*  
 Male 17, weighing 11 kilos.

Fluids analyzed.	Time.	Volume of lymph, cc.	Hemo- globin.	Total solids, gm. per 100 cc.	Chlorides, mg. per 100 cc.	Calcium, mg. per 100 cc.		Phosphorus, mg. per 100 cc.		Sugar, mg. per 100 cc.		Non-protein N, mg. per 100 cc.		Protein N, gm. per 100 cc.	
Serum.....	7.30		100	8.2	395	10.4	4.2	4.2		126		27.0		0.90	
	8.00														
Serum.....	10.00		100	8.1	393	10.4	4.3	4.3		124		27.2		0.90	
Lymph.....	11.00-1.00	20		5.3	413	9.2	3.6	3.6		124		27.0		0.57	
Serum.....	1.00		100	8.2	390	10.3	4.2	4.2		125		27.3		0.91	
Lymph.....	1.00-3.30	20		5.2	407	9.2	3.6	3.6		123		26.4		0.57	
Serum.....	3.30		100	8.2	384	10.3	4.4	4.4		120		26.3			
Lymph.....	3.30-4.30	10			392	9.3	3.8	3.8		128					
Serum.....	4.30		100		378	10.5	4.3	4.3		117					

Beginning at 11 o'clock, 200 cc. of warm water were given hourly per rectum.

	Total solids.	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.	Protein N.
	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
Serum.....	8.3	392	10.4	4.3	123	27.2	0.9
Lymph.....	5.2	413	9.2	3.6	124	27.0	0.57

The similar relationship in the values for total solids and protein nitrogen of the serum and the lymph indicates that the proteins play the largest part in determining the comparative content of solids in the two fluids.

*Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia after Exclusion of Kidney Function.*

In the present investigation, kidney function was excluded by ligation of the ureters, renal veins, and arteries so that when the kidneys failed to carry out their function in the formation of urine, catabolites accumulated in the blood. The data of an experiment, typical of the two performed, are summarized in Table III. They show that comparable consequent alterations in the concentrations of the constituents were exhibited in the serum and in the lymph. The most marked changes occurred in the content of non-protein nitrogenous substances and phosphorus which gradually increased in both fluids. The increments of these constituents were almost identical in the serum and in the lymph in a dog 6 hours after kidney exclusion as exemplified in the following figures calculated from the data presented in Table III.

Increments in:	Phosphorus.	Non-protein N.
	mg. per 100 cc.	mg. per 100 cc.
Serum.....	4.9	14.2
Lymph.....	4.7	13.5

These values illustrate the tendency to correspondence in the variations between the composition of the serum and the lymph. They show that when catabolites accumulate after occlusion of the renal circulation, they are distributed comparably between the blood and the lymph. Interchanges of diffusible constituents must thus take place promptly between the two fluids.

TABLE III.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Aneytal Anesthesia. Kidney Function Excluded by Ligation of Ureters, Renal Veins, and Arteries.*  
Male 28, weighing 17 kilos.

Fluids analyzed.	Time.	Volume of lymph. cc.	Hemoglobin.	Chlorides.	Calcium.		Phosphorus.		Sugar.	Non-protein N. mg. per 100 cc.	Protein N. gm. per 100 cc.
				mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
Serum.....	9.00										
	10.00		100	395	10.9	4.3	118		28.1	0.93	
	10.30										
Lymph.....	11.45-12.30	15		401	9.6	5.3	117				
Serum.....	12.30										
Lymph.....	12.30-1.30	15	117	385	10.8	6.1	112		33.3	0.92	
Serum.....	1.30			395	9.6	6.6	114		34.3	0.66	
Lymph.....	1.30-3.00	18	117	385	10.8	7.1	100		36.6	0.93	
Serum.....	3.00			394	9.6	7.5	100		38.1	0.65	
Lymph.....	3.00-5.00	20	117	388	10.9	8.1	100		38.3	0.94	
Serum.....	5.00		123	394		8.3	99		41.5	0.67	
				390		9.2	100		42.3	0.96	

*Composition of Blood Serum and Thoracic Lymph of Dogs after Disturbances of Carbohydrate Metabolism.*

Marked hypoglycemia can readily be brought about through the use of insulin; and hyperglycemia can be produced by anesthesia with morphine-ether or with alcohol, as well as by the introduction of glucose solution. Disturbances in carbohydrate metabolism affect not only the content of sugar in the blood but also its phosphorus content. It has been found in the present investigation that alterations in the concentrations of these two constituents in the blood are always reflected in the lymph.

*Injection of Insulin.*—The data presented in Table IV are representative of two experiments and show the effect of the injection of insulin. The fall in the content of sugar ensued more rapidly in the serum than in the lymph. Although the value for the concentration of sugar in the serum was lower than that in the lymph, the latter represents the average concentration over a period of  $2\frac{1}{2}$  hours; hence it probably was not the lowest sugar concentration reached in the lymph. Along with the hypoglycemia, a decrease in the phosphorus content occurred in the serum and the lymph. The values for non-protein nitrogen remained unchanged. There was a decrease in the chloride content of both the fluids.

*Injection of Glucose Solution.*—In Table IV the record shows that after the sugar content of the serum and the lymph had been greatly decreased following the injection of insulin, an intraperitoneal injection of glucose resulted in a rise in the concentrations of both sugar and phosphorus in the two fluids. The increase of sugar in the lymph exceeded that in the blood.

*Anesthesia with Morphine-Ether.*—The changes in the concentrations of some of the constituents of the blood serum and the lymph with the progress of morphine-ether anesthesia, as demonstrated in at least twelve animals, are detailed in the data of a typical experiment in Table V. The effect of morphine was shown in a slight hyperglycemia accompanied by a decrease in the concentrations of phosphorus and non-protein nitrogen in the blood. With the administration of ether, a steadily increasing hyperglycemia followed and was paralleled in the sugar content of the lymph. There was an augmentation in the content of

TABLE IV.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia. Intravenous Injection of Insulin.*  
*Intraperitoneal Injection of 10 Per Cent Glucose Solution.*  
 Female 18, weighing 17 kilos.

Fluids analyzed.	Time.	Volume of lymph.	Hemoglobin.	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.
		cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
	12.00			Amytal intraperitoneally.				
Serum.....	3.00		100	382	10.4	4.4	140	27.0
Lymph.....	3.30-4.30	10		392	9.0	3.6	143	27.3
Serum.....	4.30		100	380	10.3	4.2	139	27.2
	4.45			10 cc. insulin solution intravenously (200 units).				
Lymph.....	4.30-5.30	10		387	9.0	3.2	107	27.4
Serum.....	5.30		111	378	10.4	3.4	51	27.3
Lymph.....	5.30-8.00	20		388	9.0	3.1	60	
Serum.....	7.30		106	370	10.2	3.9	32	
	8.30		200 cc. 10 per cent glucose solution intraperitoneally.					
Lymph.....	8.30-9.30	15		358	9.2	4.1	546	
Serum.....	9.30		108	348	10.4	5.1	348	

TABLE V.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Morphine and Ether Anesthesia.*  
 Male 6, weighing 24 kilos.

Fluids analyzed.	Time.	Volume of lymph.	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.
		cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Serum.....	7.00		404	10.6	4.3	120	29.5
	7.30		120 mg. morphine sulfate subcutaneously.				
Serum.....	8.30		400	10.7	3.2	136	26.6
	9.00			Ether by insufflation.			
Serum.....	10.30		387	10.6	5.8	258	24.6
Lymph.....	10.30-11.30	50	402	9.2	6.5	272	25.1
Serum.....	11.30		382	10.5	7.2	295	
Lymph.....	11.30-12.30	40	386	9.2	8.1	385	
Serum.....	12.30		380	10.6	9.3	380	



TABLE VI.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Alcohol Anesthesia.*  
 Male 21, weighing 19 kilos.

Fluids analyzed.	Time.	Volume of lymph. cc.	Hemo- globin.	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non- protein N.
				mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Serum.....	7.30		100	403	10.3	4.5	127	30.0
	8.00			800 cc. 10 per cent alcohol by stomach tube.				
Serum.....	9.00			400	10.4	2.7	130	29.7
	10.30			400 cc. 10 per cent alcohol by stomach tube.				
Serum.....	10.45		111	380	10.4	3.6	134	30.6
Lymph.....	12.00-1.00	25		368	9.3	4.1	214	32.1
Serum.....	1.00		130	358	10.5	5.0	176	34.6
Lymph.....	1.00-2.00	25		353	9.4	6.0	263	37.1
Serum.....	2.00		139	346			300	39.2
	2.05					7.9		
						Animal died.		

phosphorus of the serum and the lymph. The increments of phosphorus found for both the serum and the lymph in the present investigation are in essential agreement with the recent observations of Stehle and Bourne (6) with regard to the influence of ether anesthesia on the blood alone.

*Anesthesia with Alcohol.*—Table VI gives data of one of the three experiments with alcohol. During the preliminary period after the administration of alcohol the only noteworthy change was a decrease in the content of phosphorus in the serum. This was accompanied by a marked temporary diuresis. Presently the phosphorus concentration of the serum began to rise. When the first sample of lymph was collected, the value for phosphorus was already notably high. The phosphorus content of the serum and the lymph continued to increase. There was also an augmentation of sugar in both fluids. The high values for hemoglobin and for protein nitrogen of the blood at the end of the observation indicate an unmistakable blood concentration. By comparison with data reported in other experiments, it is evident that the lymph showed an increase in protein nitrogen, an index of concentration. Apparently, therefore, both blood and lymph reflect loss of water. This concentration in the two fluids is almost sufficient to account for the somewhat augmented figures for non-protein nitrogen. The increases in the values for phosphorus and sugar of the serum and the lymph are on the other hand too large to be explained solely by loss of water.

Comparison below of the data detailed in Tables II to VI shows that alterations in the concentrations of sugar and phosphorus in the serum and the lymph are fairly consistent in the two fluids.

With disturbances of carbohydrate metabolism resulting in either hypoglycemia or hyperglycemia, fairly comparable alterations in the concentrations of sugar in the lymph follow. Whenever large increases or decreases in the content of sugar in the two fluids were exhibited, the values for sugar were always larger in the lymph than in the serum, although under "normal" conditions the sugar concentrations are about the same in the two fluids. It is possible that these differences might have been smaller had arterial blood been used instead of venous samples. In the case of phosphorus in the serum and the lymph, the rela-

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tionships of the values in the two fluids are for the most part quite comparable.

	"Normal."		"Abnormal,"			
	After amytal. Table II.	After insulin intravenously. Table IV.	After glucose intraperitoneally. Table IV.	After morphine-ether. Table V.	After alcohol. Table VI.	After exclusion of kidney function. Table III.
Sugar (mg. per 100 cc.).						
Serum*.....	123	42	348	338	238	100
Lymph.....	124	60	546	385	263	99
Phosphorus (mg. per 100 cc.).						
Serum*.....	4.3	3.7	5.1	8.3	6.5	8.7
Lymph.....	3.6	3.1	4.1	8.1	6.0	8.3

\* The serum figures are averages of the values for the blood samples taken immediately before and after the periods of collection of lymph.

### *Composition of Blood Serum and Thoracic Lymph of Dogs after Interferences with Calcium Metabolism.*

The experiments which demonstrate that it is possible to initiate and control fluctuations in the content of the calcium of the blood represent some of the outstanding recent contributions to physiology. After the parathyroids are extirpated, the calcium content of the blood decreases (*e.g.* MacCallum and Vogel (7), Salvesen (8)). If an extract of parathyroid glands is injected into either normal or parathyroidectomized animals, a condition of hypercalcemia may develop (*e.g.* Collip (9)). After the oral administration of solutions of a number of calcium salts, a rise in the calcium content of the serum ensues (Hjort (10)). In the present investigation hypocalcemia was produced by thyroparathyroidectomy, and hypercalcemia by the subcutaneous injection of parathyroid extract. The calcium content of the blood was increased by administration of a saturated solution of calcium lactate by stomach tube. The effects on the composition of the serum and the lymph of the various physiological alterations just recited are summarized from illustrative experiments in Tables VII to IX.

TABLE VII.  
*Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia. Complete Thyroparathyroidectomy.*  
 Male 26, weighing 12.5 kilos.

Fluids analyzed.	Date.	Time.	Volume of lymph. cc.	Hemoglobin.	Calcium. mg. per 100 cc.	Phosphorus. mg. per 100 cc.	Non-protein N.		Protein N. gm. per 100 cc.
							mg. per 100 cc.	mg. per 100 cc.	
Serum.....	1928 Jan. 7	2.30 p.m.		100	11.0	4.5	32.1	0.92	
		2.45		65 mg. morphine sulfate subcutaneously followed in 1 hr. with ether by insufflation.					
Serum.....	Jan. 8	4.00		Thyroparathyroidectomy.					
		10.00 a.m.		100	8.7	5.0			1.00
		12.30		Amytal intraperitoneally.					
Serum.....		4.00		100	7.9	5.1	37.5	1.03	
Lymph.....		5.00-8.00	15		6.8	7.8	37.8	0.67	
Serum.....		8.00		122	6.8				
Lymph.....		8.00-11.00	15		6.4	10.8	56.3	0.73	
Serum.....		11.00		128	6.4				

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*Thyroparathyroidectomy.*—In the thyroparathyroidectomized animal reported in Table VII, the concentration of hemoglobin in the blood remained constant during the initial development of hypocalcemia and then increased as the calcium content continued to decrease. The content of calcium in the serum had fallen considerably 16 hours after the parathyroids were removed and continued to decrease, reaching the low value 6.4 mg. per 100 cc., at the end of a period of 31 hours. At this time the concentration of calcium in the lymph was 6.4 mg. per 100 cc. Thus the values for calcium of the serum and the lymph during the last 6 hours of the period of observation are identical. With the development of hypocalcemia, a steady rise occurred in the protein nitrogen of the two fluids. Concentration, *i.e.* water loss from the circulation, apparently occurred. This was clearly manifested in the blood from which it was impossible during the last few hours to separate adequate samples of serum for analysis. Examination of the lymph during this period, however, showed an increase in the concentrations of the phosphorus, non-protein nitrogen, and protein nitrogen.

*Injection of Parathyroid Extract.*—The experiment reported in Table VIII is typical of two and shows that at the end of 18 to 24 hours after the injection of parathyroid extract, the calcium content of the serum had probably increased to a maximum. The calcium concentration of the lymph was already high when the first sample was collected, and showed only a slight further increase during the period of observation. At the end of the experiment the augmentation of calcium was comparable in the two fluids. In addition to the increase in the calcium content, a rise occurred in the phosphorus and non-protein nitrogen concentrations of the serum and the lymph. The values for non-protein reached a maximum and remained at a constant equal level in both fluids for the last few hours of the experiment. Hemoglobin and protein nitrogen decreased in concentration in the serum. Apparently a dilution occurred in the blood which suggests that the actual rise in the content of the constituents reported was even greater than the increases in the values indicate. The separation of adequate serum samples was made without difficulty. No symptoms of diarrhea or vomiting occurred.

TABLE VIII.

*Composition of Blood Serum and Thoracic Lymph of a Dog under Amytal Anesthesia. Subcutaneous Injection of Parathyroid Extract.*

Male 25, weighing 14.5 kilos.

Fluids analysed.	Date.	Time.	Volume of lymph.	Hemoglobin.	Calcium.	Phosphorus.	Non-protein N.	Protein N.
	1925		cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
Serum.....	Dec. 10	5.00 p.m. 7.00		100	11.0	4.9	27.0	0.89
Serum.....	Dec. 11	7.45 a.m. 9.00		10 cc. parathyroid extract subcutaneously. 90	16.4	6.2	26.5	0.74
Lymph.....		11.30-1.00	12		Amytal intraperitoneally.			
Serum.....		12.00		89	15.9	6.6	26.6	0.53
Lymph.....		1.00-3.00	15		17.6	6.9	31.2	0.75
Serum.....		3.00		87	16.1	6.6	31.5	0.52
					17.8	6.9	31.0	0.76

Beginning at 12 o'clock, 200 cc. of warm water were given hourly per rectum.

TABLE IX.  
*po. Blood S. and Thoracic Lymph of Dog under Morphine and Ether anesth. In*  
*of Saline Saturated Calcium Lactate Solution Stoma. Tube.*

Male 13, weighing 12 kilos.

Fluids analyzed.	Time.	Volume of lymph. cc.	Rate of flow of lymph. cc. per hr.	Clotting time of lymph. min.	Chlorides. mg. per 100 cc.	Calcium. mg. per 100 cc.	Phosphorus. mg. per 100 cc.	Sugar. mg. per 100 cc.
Lymph.....	9.00	30	60	15	386	9.4	6.1	380
Serum.....	12.00-12.30							
Serum.....	12.30							
Lymph.....	12.45	30	180	500 cc. saline intravenously. 60	444	8.7	5.6	385
Serum.....	12.50-1.00							
Serum.....	1.00							
Lymph.....	1.30	40	30 gm. calcium lactate in saturated solution by stomach tube. 40	5	466	10.7	6.6	295
Serum.....	1.30-2.30							
Serum.....	2.30							
Lymph.....	2.30-3.30	20	20	5	425	12.2	7.1	300
Serum.....	3.30							
					418	13.6	10.4	

*Administration of Calcium Lactate Solution.*—After the introduction of calcium lactate solution in four different animals, the concentration of calcium increased in the serum and the lymph as exemplified in the record of a typical experiment detailed in Table IX. With the absorption of the calcium lactate and the subsequent increases of calcium in both fluids, the rate of flow of lymph decreased and it clotted unusually rapidly. No change was observed in the clotting time of the blood.

Comparison below of the data presented in Tables II, VII, VIII, and IX shows that deviations from the "normal" calcium content of the blood are usually followed by comparable variations in the concentrations of the lymph calcium.

	"Normal,"	"Abnormal,"			
	After amygdal.	17 hrs. after thyroparathyroidectomy.	19 hrs. after parathyroid extract subcutaneously.	2 hrs. after calcium lactate solution by stomach tube.	10 min. after saline intravenously.
	Table II.	Table VII.	Table VIII.	Table IX.	Table IX.
Calcium (mg. per 100 cc.).					
Serum*.....	10.4	6.6	17.7	12.9	9.4
Lymph.....	9.2	6.4	16.1	11.4	8.0

\* The serum figures are averages of the calcium values for blood samples taken immediately before and after the periods of collection of lymph.

When alterations in the calcium content of the blood are produced, the values of this element in the lymph are likewise changed. In all of the conditions except that of hypocalcemia following parathyroidectomy, the altered concentrations of calcium show a parallelism to what exists in the serum and lymph of the "normal" animals. In the thyroparathyroidectomized dog, the values show a greater decrease in the concentration of the serum than of the lymph calcium.

Changes, which were observed in the concentrations of some of the other constituents of the blood serum and the lymph, when disturbances in the calcium metabolism occurred, were likewise paralleled in the two fluids.



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### *General Considerations Regarding the Relationships between Composition of Blood Serum and Thoracic Lymph.*

In animals under essentially "normal" conditions, *i.e.* under amytal anesthesia, the compositions of the serum and the lymph indicate that definite quantitative relationships exist between the concentrations of the constituents of the two fluids. Calcium, phosphorus, and protein are present in greater concentrations in the serum than in the lymph; whereas the concentration

TABLE X.

*Comparisons of Concentrations of the Constituents in Blood Serum and Thoracic Lymph of Dogs under "Normal" and "Abnormal" Conditions.*

Ratio	Concentration of constituents of serum Concentration of constituents of lymph					
	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.	Protein N.
"Normal."						
After amytal.....	0.95	1.13	1.19	0.99	1.007	1.58
"Abnormal."						
After exclusion of kidneys.....	0.98	1.13	1.04	1.00	0.98	1.42
" thyroparathyroidectomy....		1.03			0.99	1.54
" injection of parathyroid extract.....		1.10	1.04		0.99	1.45
After administration of calcium lactate solution.....	0.98	1.13	1.14	1.01		
After administration of glucose solution.....	0.97	1.13	1.24	0.60	0.99	
After anesthesia with morphine-ether.....	0.98	1.12	1.02	0.88	0.98	
After anesthesia with alcohol....	0.98	1.12	1.09	0.89	0.98	1.57
" injection of insulin solution.	0.96	1.14	1.18	0.70	1.00	

of chloride is less in the serum than in the lymph. Not only the sugar but also the non-protein nitrogenous substances on the other hand occur in almost identical concentrations in the two fluids. Whenever changes in the chemical composition of the blood are produced as a result of experimental manipulations causing "abnormal" conditions in the animals, comparable alterations are exhibited in the concentrations of the constituents of the lymph so that characteristic relationships are maintained

between the compositions of the serum and the lymph. Values giving the ratios of the estimated constituents of the two fluids, summarized in Table X, show that with few exceptions the constant relations exhibited between the concentrations of the constituents of the serum and the lymph of "normal" animals likewise hold for those under "abnormal" conditions.

The ratios for the non-protein nitrogenous substances are very constant in all of the conditions investigated. The values for the ratios of the other constituents, although for the most part showing fairly constant figures, do differ to some extent—the greatest variation existing in the relationship of the sugar concentrations of the serum and the lymph.

The differences in concentration of the protein in the serum and the lymph respectively remain conspicuous as is shown in their constant ratio under all conditions. The proteins are for the most part non-diffusible and therefore do not pass the semi-permeable membrane represented by the vessel walls.

The variation in the comparative distribution of the inorganic ions and the protein in the serum and the lymph suggests that a difference in the electrical potential of the two sides of the semi-permeable membrane, represented by the vessel walls, may play a part in determining the characteristic concentrations of the constituents of the two fluids. According to the Donnan equilibrium, if the protein is present as the anion, wherever the protein concentration is higher, the concentration of chloride ions tends to be lower, and the concentration of calcium ions tends to be higher. These conditions are demonstrated in the relations of the constituents in the serum and the lymph.

The almost constant ratios of all of the estimated constituents of the serum and the lymph both in "normal" and "abnormal" conditions show that fairly fixed modes of exchange exist between the blood and the lymph.

## 210 Chemical Composition of Blood and Lymph

### SUMMARY.

It was found that by the use of the anesthetic amytal the composition of the serum and the lymph of the dog remained almost constant for periods of 6 to 18 hours. Animals under such anesthesia were therefore considered to be essentially "normal."

A study was made of the comparative chemical composition of blood serum and thoracic lymph of the dog under relatively "normal" conditions, *i.e.* under amytal anesthesia, and under "abnormal" conditions, *i.e.* after (1) exclusion of kidney function, (2) interferences with carbohydrate metabolism, and (3) experimental procedures causing changes in the content of calcium of the body fluids.

Under "normal" conditions the values for the estimated constituents of the serum and the lymph of the dog are as follows:

	Total solids.	Chlorides.	Calcium.	Phosphorus.	Sugar.	Non-protein N.	Protein N.
	<i>gm. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>gm. per 100 cc.</i>
Serum.....	8.3	392	10.4	4.3	123	27.2	0.9
Lymph.....	5.2	413	9.2	3.6	124	27.0	0.57

Experimental interferences with physiological functions of the animals were found, in almost all of the conditions investigated, to produce comparable variations in the make-up of the serum and the lymph. The concentrations of phosphorus and non-protein nitrogenous substances increased in the serum and the lymph after occlusion of the renal circulation. Sugar and phosphorus increased in both fluids after anesthesia with morphine-ether or alcohol and after injection of glucose solution; their concentrations in the two fluids decreased after injection of insulin solution. The content of calcium increased in the serum and the lymph after injection of parathyroid extract and after the introduction of calcium lactate solution, and decreased in both fluids after thyroparathyroidectomy.

In both "normal" and "abnormal" conditions, characteristic interrelationships exist between the concentrations of the constituents of the serum and the lymph.

It is apparent from these studies that interchanges take place between the blood and the lymph whenever fluctuations in the concentrations of the constituents occur, and that diffusible substances pass easily and rapidly at all times between the blood, the lymph, and the tissues.

Grateful acknowledgment is made of the helpful cooperation of Professor George R. Cowgill and the assistance of Mr. Robert A. Frisch.

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## ISOLATION OF THYROXIN.

By EDWARD C. KENDALL.

*(From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota.)*

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In 1919 a method for the isolation of thyroxin from thyroid material was described. The method was based upon the destruction of the protein material in the thyroid gland by means of sodium hydroxide. Acidification of this solution precipitated a small amount of material which contained from 30 to 50 per cent of the total iodine. By treatment with barium hydroxide certain impurities were removed and the iodine-containing compound was concentrated in a fraction which was always acid-insoluble, but which changed in its solubility in barium hydroxide. In the presence of certain impurities barium hydroxide exerted a solvent action upon almost the entire portion of the acid-insoluble material. As the purification was continued, thyroxin concentrated in the barium-insoluble fraction. Eventually it was crystallized as its sodium salt, which was insoluble in the presence of sodium chloride, or sodium hydroxide, and its final purification was effected through precipitation from alkaline alcohol, after acidification with acetic acid. The method was laborious and time-consuming, and resulted in the distribution of material over a large number of precipitates, and called for the retreatment of the various fractions separated. It had been noted, although not published in detail, that thyroid glands obtained in the middle west of the United States contained much less thyroxin in the winter than those obtained in the summer, and that certain samples of thyroid material responded to the method with a much higher yield of pure thyroxin and a lower yield of the other iodine-containing fractions.

In 1926 Harington published a method for the isolation of thyroxin from the thyroid gland. His method consisted in the use

of barium hydroxide instead of sodium hydroxide. The desiccated thyroid was first treated with about an equal weight of 10 per cent crystalline barium hydroxide. The barium hydroxide-insoluble fraction, precipitate B, was filtered out and the filtrate was acidified. The acid-insoluble material, precipitate A, was treated for 18 hours with 40 per cent barium hydroxide. Harington found that all the thyroxin which could be isolated was present as an insoluble barium residue after treatment with 40 per cent barium hydroxide solution, and he states that the barium-soluble portion "is of no further value as regards thyroxin." The yield from 500 gm. of desiccated thyroid which contained 0.55 per cent of iodine was 0.4 gm. of thyroxin from the barium-soluble portion of the 10 per cent barium hydroxide extract, and from the barium residue, after the 10 per cent barium hydroxide treatment (precipitate B) by an identical treatment to that given precipitate A after precipitate B had been decomposed with sodium hydroxide and sulfate and had been precipitated by acid, he was able to separate 0.9 gm. from 2000 gm. This gave a total of 0.12 per cent thyroxin, which separated in crystalline form from an alkaline alcohol solution with acetic acid.

This yield is many times that obtained by the use of my method as originally published, and the method proposed by Harington appeared to be a distinct improvement over the original work. However, it seemed difficult to reconcile the chemical properties of thyroxin described by Harington with certain facts concerning the behavior of thyroxin which I had observed throughout the attempts to isolate this compound. The point of greatest difference is the statement that the 40 per cent barium hydroxide solution "is of no further value as regards thyroxin" after the 18 hour treatment. Similar treatment of thyroid material which had been carried out in this laboratory previous to Harington's publication had shown that whether thyroxin was soluble or not in the presence of barium hydroxide did not depend upon the concentration of the hydroxide, but did depend upon the impurities which were present.

In order to carry out certain investigations with thyroxin it became necessary to separate some more of this substance. For this purpose fresh thyroid glands were obtained, and the old method using sodium hydroxide was used. A few gm. of thyroxin

were isolated, but the yield was not satisfactory. For this reason and with the object of investigating the details of the method published by Harington, the barium hydroxide treatment was applied to fresh thyroid glands, and immediately insurmountable difficulties were encountered. No thyroxin could be isolated from the glands which were treated with barium hydroxide, and this suggested a more careful investigation of the method described by Harington.

For this purpose desiccated thyroid was obtained which contained 0.34 per cent iodine. This iodine content was 62 per cent of the iodine content of the desiccated thyroid used by Harington, and theoretically should give approximately 62 per cent of the yield of thyroxin which he obtained. All the details of the method outlined by Harington were followed with scrupulous care, and in addition to this the iodine content of the various solutions was also determined. 500 gm. of desiccated thyroid were heated for 6 hours in 5 liters of water containing 500 gm. of crystalline barium hydroxide. The solution was allowed to stand overnight, and then was filtered, and the filtrate made acid with hydrochloric acid. A light yellow voluminous precipitate settled to the bottom of the vessel. The solution was allowed to stand for a few hours; the precipitate was filtered and was then dissolved in ammonia and placed in 250 cc. of water containing 100 gm. of crystalline barium hydroxide. This solution was heated for 18 hours at 100°. It was then filtered through a hot suction funnel in order to prevent the crystallization of barium hydroxide. The barium residue, precipitate C, was suspended in water containing 1 per cent sodium hydroxide and sodium sulfate. This solution was boiled until the barium salts were all decomposed and the barium was present as barium sulfate. The solution was filtered and was then heated to boiling and made acid with 50 per cent sulfuric acid, following the precise conditions suggested by Harington. The precipitate was not further purified. This procedure was carried out with four 500 gm. lots of desiccated thyroid. The four precipitates from precipitate C were dried and weighed; the weights were as follows: 0.130, 0.200, 0.110, and 0.150 gm., a total of 0.590 gm. The barium residue from the 10 per cent barium hydroxide treatment, precipitate B, was saved until four lots had been accumulated, as suggested by Harington. This was then treated with sodium hy-



dioxide and sodium sulfate with a disappointing result. A thick, gelatinous solution was obtained, which would support a heavy glass stirring rod even when heated to boiling. It was unfilterable, would swell to the full capacity of any vessel in which it was placed, and was so different from the solution which one would visualize after reading Harington's description of this step in the method that it became apparent that the material used by Harington had very different properties from the desiccated thyroid under investigation. No thyroxin could be isolated from the barium residue of the 10 per cent barium hydroxide solution by the method outlined by Harington.

The four precipitates obtained from the portion soluble in 10 per cent barium hydroxide were combined and dissolved as Harington suggests in 20 cc. of *N* sodium hydroxide, to which alcohol was added to approximately 80 per cent strength. A small amount of tar was separated from the solution which was then acidified with 10 cc. of 30 per cent acetic acid. No thyroxin separated immediately, and after the solution had stood overnight it was filtered. The weight of the insoluble material was less than 10 mg. and this was not thyroxin. By a strict adherence to Harington's method no thyroxin could be isolated from the desiccated thyroid.

Harington stated that the 40 per cent barium hydroxide solution after removal of the barium-insoluble material "is of no further value as regards thyroxin," and that it was all present in the barium residue. However, examination of the 40 per cent barium hydroxide solution, which I shall call solution D, after removal of the barium residue indicated that some thyroxin was present in the solution. In order to determine this the solution was allowed to cool and the barium hydroxide which separated in crystalline form was removed by filtration. The small amount of barium hydroxide remaining in solution was neutralized with hydrochloric acid.

A voluminous precipitate was formed in each and every barium hydroxide solution regardless of the strength of the hydroxide used. The weights of the four precipitates obtained from the four lots of the desiccated thyroid by acidifying the 40 per cent barium hydroxide, solution D, after filtering out the barium hydroxide which had crystallized, were 0.200, 0.480, 0.520, and 0.370 gm., a total of 1.570 gm.

In order to test the influence of a higher concentration of barium hydroxide 200 gm. of barium hydroxide were added in a total volume of 250 cc. instead of 100 gm. This concentration of 80 per cent barium hydroxide did not alter the amount of iodine-containing compounds in the barium residue or in the solution. The weight of the precipitate derived from the barium-insoluble portion after extraction with sodium hydroxide and sodium sulfate was 0.155 gm., and the weight of the precipitate from the barium-soluble portion was 0.300 gm. These findings agree with what had already been observed. The solubility of thyroxin in barium hydroxide depends upon the other constituents of the solution and not upon the concentration of the hydroxide.

The four precipitates obtained by acidifying the barium hydroxide, solution D, were dissolved in sodium hydroxide. The total iodine content of this solution was 522 mg. The iodine content of the alcohol solution containing the four precipitates secured by decomposing the barium residue, precipitate C, filtered from the 40 per cent barium hydroxide solutions was 294 mg. There is no evidence that all of the iodine in these two solutions was in the form of thyroxin, and it undoubtedly was not.

It was now necessary to show that pure crystalline thyroxin could be separated from both of these fractions. The alcohol solution from which thyroxin had failed to crystallize after acidification with acetic acid was made alkaline and the alcohol was removed by boiling. Ammonium chloride was added to this solution. This gave a voluminous, flocculent precipitate, which was filtered from the solution, dissolved in ammonium hydroxide and reprecipitated with ammonium chloride. This was repeated four times. The precipitate was then treated with 5 N sodium hydroxide at 100°, and on cooling the almost white crystalline sodium salt of thyroxin separated from solution. This was recrystallized again from 5 N sodium hydroxide solution. The sodium salt was separated from the hydroxide solution in a centrifuge tube. The sodium salt was then dissolved in alcohol, the solution was filtered from some tar, and when it was made acid with acetic acid thyroxin separated in crystalline form. It weighed 185 mg.

From the barium-soluble portion, solution D, thyroxin was separated by an entirely similar treatment. The weight of the

crystalline thyroxin separated from alcohol after acidifying with acetic acid was 250 mg.

The distribution and amounts of thyroxin separated when compared with Harington's work, demonstrate conclusively that desiccated thyroid prepared in England is radically different from American thyroid, and further that Harington's method does not furnish a means of separation of thyroxin from any sample of American thyroid glands so far investigated.

These results indicate a condition existing in the thyroid glands of animals, which has not been recognized up to the present and which is not comparable with any variation known in the other ductless glands caused by geographical distribution. Harington believed that the cause for the small yield of thyroxin given by my method was the 24 hours boiling in 5 per cent sodium hydroxide and, for this reason, he substituted barium hydroxide for sodium hydroxide. By using barium hydroxide on English desiccated thyroid he was able to obtain a much better yield than I had obtained when using sodium hydroxide on American desiccated thyroid and the natural inference was that the improved yield was due to the substitution of the sodium with barium hydroxide. It now appears certain that this is not the explanation. Thyroxin is stable in 5 per cent sodium hydroxide and it is not affected by atmospheric oxygen or hydrogen dioxide in alkaline solution, during the length of time necessary for its separation. Treatment of the whole thyroid gland in 5 per cent sodium hydroxide for 24 hours breaks down most of the protein material and permits thyroxin to be precipitated with acid.

The amount of iodine in the acid precipitate after sodium hydroxide hydrolysis has been found to vary with the sample of thyroid, and this can be related also to a seasonal change in the gland. Not only is there less iodine in the winter, but the amount of iodine in each gm. of acid-insoluble material is very much lower; however, the percentage of the total iodine in the thyroid material which is precipitated by acid does not change materially with the season. It was originally assumed that all of the iodine in the acid-insoluble portion was thyroxin and that failure to isolate it was due to decomposition of thyroxin during its isolation. It now appears probable that this is not the correct explanation.

There are four well established facts which bear an intimate

relation, the precise explanation of which cannot now be given. These are:

1. Desiccated thyroid will relieve hypothyroidism in proportion to its iodine content.

2. Pure, crystalline thyroxin will relieve hypothyroid conditions in the same manner and to the same extent as desiccated thyroid. It follows therefore that there is nothing else present in the thyroid gland which is necessary for the relief of hypothyroidism in so far as we know.

3. The percentage of the total iodine which is insoluble in the presence of acids after hydrolysis with sodium hydroxide is remarkably constant and for normal glands lies between 35 and 45 per cent.

4. The percentage of the total iodine-containing compounds which can be isolated in the form of crystalline thyroxin is not constant.

It varies from 0 to 14 per cent.

There are two possible explanations for the failure to isolate thyroxin in an amount which bears a constant relation to the total amount of iodine present. The first explanation is that more thyroxin is destroyed during the treatment of one sample of thyroid material than of another. This does not seem probable. Pure, crystalline thyroxin when put back into partially purified thyroxin and treated with 5 per cent sodium hydroxide can be recovered without loss. Although it is not probable that all of the thyroxin present is isolated by any method, at least most of the thyroxin which is present may be isolated by my original method. The second explanation is that when thyroxin functions in the animal organism it is still further elaborated by the addition of some other group, which changes its chemical properties, preventing its crystallization with thyroxin. If this is true, then the thyroxin which can be isolated is merely that part of the total active agent of the thyroid which happens to be present in that stage of elaboration which has the configuration of thyroxin. This assumption agrees well with all that is known concerning the physiological activity of the gland and the chemical nature of thyroxin.

The iodine-containing compounds that are acid-soluble do not possess physiological activity, and probably represent the intermediate stages in the synthesis of thyroxin from the uniodized

nucleus, but all iodine-containing compounds which are insoluble in mineral acid are probably either thyroxin or further elaborated forms of this compound. Any hypothesis stated at this time in regard to this is merely speculation, but an extensive study of the influence of geographical distribution and other variables on the percentage of the total iodine which can be separated as thyroxin is now being carried on.

When Harington undertook the isolation of thyroxin from the thyroid gland he did not know that the thyroid glands of animals varied according to their geographical distribution, and with little trouble he isolated a large amount of thyroxin which enabled him to remove the iodine from sufficient of the material to identify the nucleus. It has been and apparently always may be impossible to use the thyroid glands removed from animals of the middle west of the United States as a source of thyroxin comparable with the glands prepared in England. In order to isolate 100 gm. of thyroxin from American glands, which was the amount used by Harington in the determination of the structural formula, the equivalent of several years' work and at least 30,000 dollars would be required. Since up to the present investigation there was no possible escape from this fact, and since the only apparent escape today is the use of English glands, it was decided to prepare the triiodohydroxindole propionic acid derivatives suggested for the configuration of thyroxin, and at some future time to reduce the iodine from sufficient thyroxin to compare the organic nucleus so obtained with some of the derivatives prepared synthetically.

The work of Harington has shown that thyroxin is not an indole derivative and one may ask what was the evidence upon which the formula of thyroxin suggested in 1919 was based. The three determinations which were the basis for our belief that thyroxin was an indole derivative and that the molecular weight was 585 were the comparisons of the percentage of iodine in thyroxin with that in the sulfate salt, in the acetyl derivative, and in the ureide. The percentage of iodine in thyroxin was determined to be 65.1; the percentage of iodine in the sulfate was found to be 60.1. Assuming that the difference in iodine content was due to the sulfate salt the molecular weight of thyroxin would be 585. The iodine content of the acetyl was 60.87; and assuming the added radical, acetyl, to be 42 the molecular weight of thyroxin would

be 585. The percentage of iodine in the ureide was 60.7, and assuming the added radical to be 43 the molecular weight determined through this derivative would be 585. With the molecular weight fixed at this figure the number of carbon atoms was 11, and the number of iodine atoms was 3.

There still remains the necessity of explaining how the sulfate, the acetyl, and the ureide all indicate a molecular weight of 585. There are two possible explanations. One is that an error was made in the analytical work of each of these three derivatives. We have no reason to believe this to be true, and in fact we have recently confirmed the iodine content of the sulfate and the acetyl. The second explanation is that in addition to the radicals added to thyroxin some other substance was unknowingly added at the same time. This may be the case. If 18, the molecular weight of water, is added to the molecular weight of the three derivatives, sulfate, acetyl, and ureide of thyroxin, assuming the molecular weight of thyroxin to be 777, the percentages of iodine calculated are 60.1, 60.77, and 60.67. These calculated figures agree within experimental error with the actual determinations. Since all derivatives of thyroxin prepared before or since the publication of the work in 1919 indicate a molecular weight of 585, we did not feel any hesitancy in accepting this figure as correct. This molecular weight agreed with that required for a triiodo derivative of tetrahydrooxindole propionic acid.

The details of the synthesis and chemical properties of the large series of oxindole propionic acid derivatives which have been prepared will be published in the near future. No further discussion of the properties and configuration of thyroxin will be given at this time. I congratulate Harington on bringing to a successful close the identification and synthesis of one of the most interesting substances known.

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## GROWTH EXPERIMENTS ON DIETS RICH IN FAT.\*

BY HAROLD LEVINE AND ARTHUR H. SMITH.

(*From the Laboratory of Physiological Chemistry, Yale University,  
New Haven.*)

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The newer knowledge of the nutritive rôle of the various dietary constituents and the consequent improvement of experimental rations have made possible types of metabolism studies hitherto unattempted. Osborne and Mendel (1911) and Cowgill (1923) employing the rat and dog respectively, have demonstrated that these animals will eat mixtures of purified food materials—the so called synthetic diets—provided the vitamin requirements of the animal are satisfied.

Inasmuch as the rat can be successfully fed such mixtures of isolated food substances, it has been possible to shift the proportions of the organic nutrients—fat, protein, and carbohydrate—to extremes, without encountering obvious abnormal effects in the animal organism. Thus various investigators, namely Osborne and Mendel (1920), Frank (1922), Drummond (1921), Smith and Carey (1923), and Jackson and Riggs (1926), have been able to obtain “normal” growth in rats on diets, the energy of which was derived almost entirely from either fat, protein, or carbohydrate. In the present study, rats have grown at the normal rate from 30 gm. to 180 gm. in body weight on a ration practically devoid of preformed carbohydrate but containing 86 per cent of its calorie content in the form of fat.

In comparing the relative efficiency of fat and carbohydrate for growth, Smith and Carey (1923) were unsuccessful in their efforts to obtain prolonged growth at the normal rate in rats on a ration

\* The experimental data in this paper are taken from the dissertation submitted by Harold Levine in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1926.



containing 69 per cent of fat (86 per cent fat calories) and no preformed carbohydrate. Their rats did not grow at the normal rate after 50 days of age. These investigators, in attempting to explain their results, were inclined towards the opinion that the young animals were losing energy through the imperfect combustion of the fatty acids, the end-result being manifested in an abnormal growth rate. They noticed, however, in the case of one rat that when a diet containing 59 per cent of fat and 11.9 per cent of carbohydrate was substituted for the 69 per cent fat ration, an immediate response in the direction of a normal growth rate took place.

Using rations devoid of preformed carbohydrate and having a fat content as high as 75 per cent, Osborne and Mendel (1924) were able to secure growth in some of their rats. Their results are, therefore, at variance with those of Smith and Carey (1923). Osborne and Mendel were unable, however, to secure growth on diets in which fat represented more than eight-tenths to nine-tenths of the calorie intake. The small food intake, occasioned by the high calorie value of these high fat diets, together with the resulting inadequate protein and salt intake, was no doubt responsible for the failure of their animals to grow. Although successful growth was obtained by these investigators on rations with a fat content as high as 75 per cent, several changes were made in the regimen (such as the use of yeast and additions of casein) so that the proportionate relationship between the ingested fat and protein was altered. These experiments are, therefore, open to the criticism that the dietary factors were not kept constant throughout the experimental period. As a result of their experiments, Osborne and Mendel concluded that "in as far as carbohydrate is required for the intermediary metabolism, particularly for the metabolism of fats and the development of muscular contraction, it can be furnished endogenously throughout the period of growth to adult size."

Smith and Carey (1923) and Osborne and Mendel (1924) in their experiments employed mixtures of purified food substances. On the other hand, Frank (1922) used a high fat ration made up of a mixture of whole milk powder, butter, and cream. Calculated on a dry basis, this diet provided:

	In dry matter, per cent	In calories, per cent
Protein.....	15.0	8
Fat.....	80.0	91
Carbohydrate.....	2.7	1
Ash.....	1.2	

Excellent growth was obtained in rats with this ration. He also compared the rate of growth of these animals with the rate in two other groups of animals; i.e., a group on a high carbohydrate ration consisting of either barley, noodles, oats, or groats and a group on a diet made up of scraps, which was considered a normal ration. Frank found that the rats on the fat ration grew at a faster rate than those on the carbohydrate and the normal diets. To double, triple, and quadruple the initial weight at the start of the experiment, the animals fed the normal food required 26, 54, and 122 days, respectively, the carbohydrate-fed rats, 30, 80, and 116 days, while the animals on the fat ration made the same increases in body weight in 28, 42, and 70 days. The average initial weights of the rats in the three groups were respectively, 39, 34, and 34 gm. The results in the three groups are, however, not strictly comparable since there is a possibility that the rats on the carbohydrate diet and normal food were consuming biologically inferior proteins present in the various cereals in contrast to the better milk proteins consumed by the animals fed the fat ration.

In the experiments here reported, the attempt was made to demonstrate normal growth in rats on a high fat ration practically devoid of preformed carbohydrate and containing 86 per cent of the calories in the form of fat. Groups of rats were also placed on various diets rich in fat containing small amounts of preformed carbohydrate. Simultaneously with the above investigation the problem of calorie utilization for *growth* was studied in rats consuming, on the one hand, a diet containing a maximum of fat calories and, on the other hand, a mixture in which the energy was derived from both fat and carbohydrate. Along with these studies, the extent of fat absorption on various high fat diets was also determined.

### *I. Growth Experiments.*

Five groups of young rats—A, B, C, D, and E (see Charts I and II), weighing approximately 40 gm. at 30 to 35 days of age were

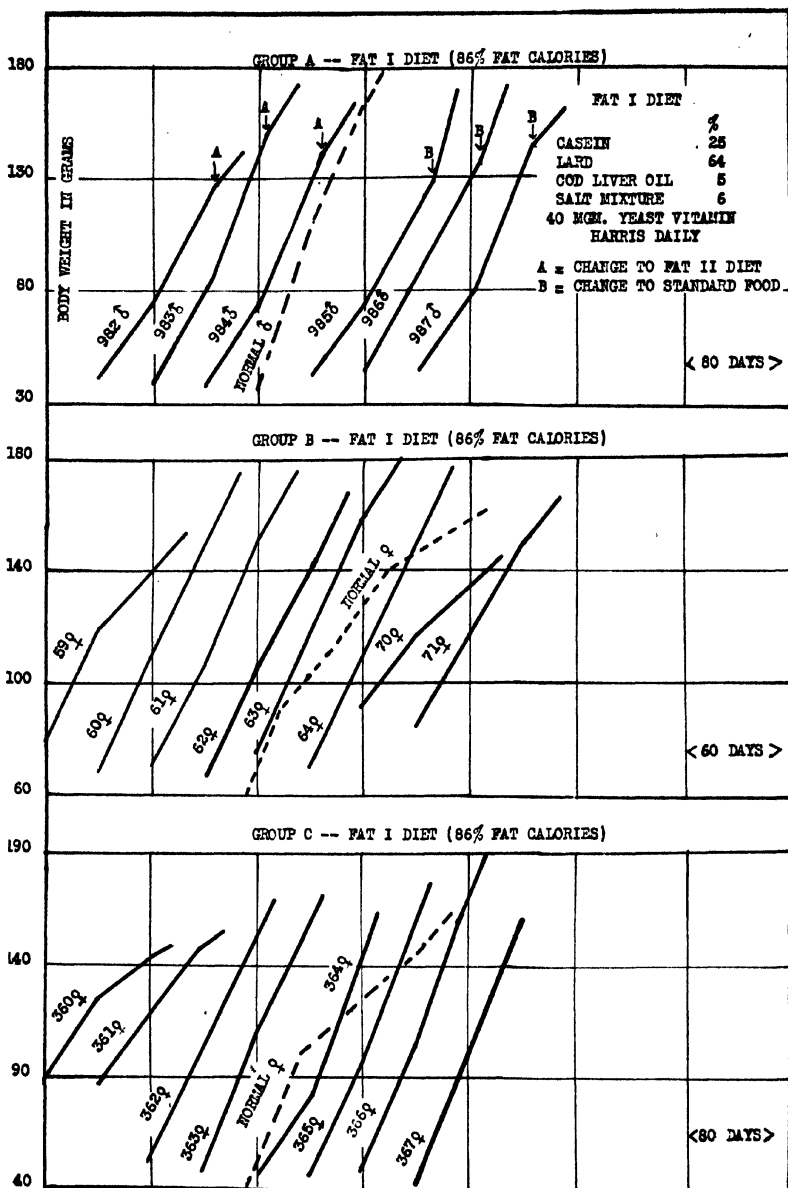


CHART I. Showing the growth of rats on a high fat (Fat I)<sup>1</sup> diet containing practically no preformed carbohydrate. Of the total calories 86 per cent are in the form of fat calories.

TABLE I.  
*Composition of Diets.*

Composition.		Calories per kilo of food.	Apportionment of total calories.	
Fat Diet I.*				
	<i>per cent</i>			<i>per cent</i>
Casein.†	25	1025	Protein.	14.0
Lard.	64	6417	Fat.	86.0
Cod liver oil.	5			
Salts.‡	6			
	—	—		—
	100	7442		100.0
Fat Diet II.*				
Casein.	23.5	964	Protein.	14.0
Starch.	11.9	488	Carbohydrate.	6.0
Lard.	54.0	5487	Fat.	80.0
Cod liver oil.	5.0			
Salts	5.6			
	—	—		—
	100.0	6939		100.0
Fat Diet III.*				
Casein.	21.7	890	Protein.	14.0
Starch.	24.1	988	Carbohydrate.	15.0
Lard.	44.0	4557	Fat.	71.0
Cod liver oil.	5.0			
Salts.	5.2			
	—	—		—
	100.0	6435		100.0
Fat Diet IV.*				
Casein.	20.0	820	Protein.	14.0
Starch.	36.2	1484	Carbohydrate.	25.0
Lard.	34.0	3627	Fat.	61.0
Cod liver oil.	5.0			
Salts.	4.8			
	—	—		—
	100.0	5931		100.0

TABLE I—*Concluded.*

Composition.		Calories per kilo of food.	Apportionment of total calories.	
Standard Diet V.*				
	<i>per cent</i>			<i>per cent</i>
Casein.	18	738	Protein.	14.0
Starch.	51	2091	Carbohydrate.	39.0
Lard.	22	2511	Fat.	47.0
Cod liver oil.	5			
Salts.	4			
	<hr/>	<hr/>		<hr/>
	100	5340		100.0

\* 40 mg. of Yeast Vitamin Powder (Harris) fed daily apart from the food.

† A fat-free product, No. 453, from the Casein Manufacturing Company, 13 Park Row, New York. It was washed with water of pH 4.6 in a centrifuge basket, then with alcohol (95 per cent) and dried in a steam drier. By this process, the protein content ( $N \times 6.25$ ) was raised to approximately 90 per cent, while the ash content was reduced to about 1 per cent.

‡ The inorganic salt mixture described by Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374).

grown on High Fat Diets I, II, and III. Table I shows the nature of the five diets employed in the investigation. The percentage composition, caloric value per kilo of food, and the apportionment of total calories are given. The diets were so planned that no matter what the caloric value of the food, the ratio of protein calories and of salt content to the total calories would be the same. The only variable dietary factors were, therefore, fat and carbohydrate. The adjustment of intake to the caloric value of the food could, therefore, be made without disturbing the actual consumption of the two indispensable constant factors; namely, the inorganic salts and the protein.

In carrying out the experimental plan three of the groups (A, D, and E) were placed on Fat Diets I, II, and III which contained respectively 86, 80, and 71 per cent of the total calories in the form of fat. Fat Diet I contained the highest amount of fat compatible with the fulfilment of the protein and salt requirements of the rat and was practically devoid of preformed carbohydrate. Small amounts of the latter, however, were included in Fat Diets II and III.

Previous experience (Smith and Carey, 1923) had indicated that better growth would be obtained when a small amount of carbohydrate was included in the ration. However, in the present experiments all the rats in the three above mentioned groups

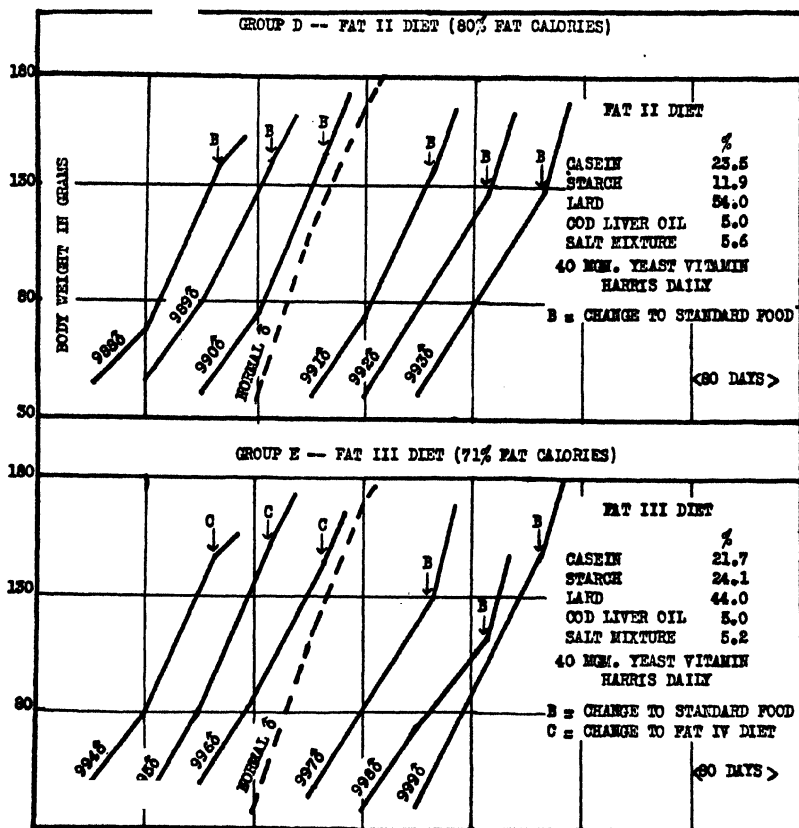


CHART II. Showing the growth of rats on high fat diets (Fat Diets II and III) containing small amounts of preformed carbohydrate. Fat Diet II contains 80 per cent of the total calories in the form of fat while Fat Diet III contains 71 per cent.

exhibited a rate of growth only slightly below normal. Chart I, Group A, and Chart II, Groups D and E, show the results of these experiments. Various attempts were subsequently made to accelerate the rate of growth. Thus, increasing the vitamin B

supplement and making frequent renewals of the rations in the food cups in the cages were without effect. In some cases, substitution with various fat diets (including Fat Diet IV), containing larger amounts of preformed carbohydrate, did not result in a change in the rate of growth. Replacement with Standard Diet V seemed to increase the growth rate slightly.

It seemed possible that in the experiments just mentioned some unexplainable disturbing factor—perhaps hereditary in nature—was operating, since Fat Diet IV which, in composition, closely approximated Standard Diet V on which good growth has been repeatedly obtained in this laboratory, did not increase the rate of growth. It was decided, therefore, to repeat attempts to obtain normal growth on Fat Diet I. Accordingly another group of animals (Chart I, Group B) was placed on this diet, and good growth, in some cases better than normal, occurred. Still a third series of rats was started on Fat Diet I and here, again, good growth was obtained (Chart I, Group C). *Obviously, therefore, the above results, successful on the two latter series of rats, afford sufficient experimental evidence that normal growth from 30 gm. to 180 gm. of body weight is possible on a high fat ration practically devoid of preformed carbohydrate and containing 86 per cent of the total calories in the form of fat, providing the protein and salt requirements of the animal are satisfied.*

Section of the livers of Rats 63 and 64 when stained with Sudan III and examined under the microscope were devoid of fat droplets and showed a normal histological picture. Rats 63 and 64 had been on the high fat ration for a period of 83 days. In this connection, the results of Chalатов (1912) are of interest. This investigator fed rats on a daily ration consisting of one egg yolk mixed with milk. Fat droplets appeared in the liver and continued to increase in number for 2 months, after which there was a decrease. After 4 months of feeding this ration, the fat droplets disappeared completely from the liver.

The results obtained in the present investigation are in harmony with those of Osborne and Mendel (1924) and are in accord with their conclusion that "in as far as carbohydrate is required for the intermediary metabolism, particularly for the metabolism of fats and the development of muscular contraction, it can be furnished endogenously throughout the period of growth to adult size."

## II. Utilization of Calories in Growth.

Most of the experimental work bearing on the problem of fat and carbohydrate utilization has been concerned with the relative efficiency of these nutrients as a source of muscular energy, *i.e.* in exercise. Only a few investigators, however, have attempted to determine the relative efficiency of these "proximate principles" in supplying the energy demand for *growth*. Will calories derived largely from fat function as efficiently as carbohydrate calories in *growth*, one of the most severe requirements made on the animal metabolism?

Although a strict comparison of fat and carbohydrate calories in growth has not been attempted, a few workers have sought to determine the growth-promoting power of various diets employed in their experiments. Thus, Hopkins (1912) found that the *absolute* food consumption of rats growing rapidly on a synthetic diet (similar to Standard Diet V) to which a milk supplement was added, was greater than that of those growing slowly (or not growing) upon the basal dietary alone; *but the consumption per unit of live weight* was nearly the same in comparable groups. This investigator calculated from data of food intake the energy consumption of rats in terms of *calories per 100 gm. of live weight*.

Drummond (1918) sought to ascertain whether the food consumption of rats is affected by the caloric value of the diet when the water-soluble factor B is absent. One group of animals was fed a high calorie diet, having a value of 5.06 calories per gm. of food, while another group consumed a low calorie ration having a value of 3.04 calories per gm. Whether vitamin B was present or not, it was found that the *caloric intakes per 100 gm. of live weight* in the two groups of animals were comparable throughout the period of the experiment.

Similar results were obtained by Macallum (1919) who in studying the relation of vitamins to growth of young rats made calculations of the *energy intake per 100 gm. of live weight*. He found that the caloric requirements of the rat display a tendency to diminish with the increasing duration of the experiment. This finding is also in accord with that of Hopkins (1912).

Smith and Carey (1923) appear to have been the first to make a study of the relative efficiency of fat and carbohydrate calories *in growth*. In experiments on rats, they employed, besides a



standard balanced ration, extreme fat and carbohydrate diets. In all of these rations, the ratio of salt and of protein calories to the total calories was the same, so that the only variables in the diets were the fat and carbohydrate. They compared the caloric consumption of these diets in rats making gains of 19 to 21 gm. in body weight, between 40 and 50 days of age. They were unable to choose a longer range on the growth curve—obviously more desirable—because the animals on the high fat ration ceased to grow at the normal rate after 50 days of age. These investigators concluded from their experiments that, "Rats making comparable increments of growth in the same time consume nearly the same number of calories from diets high in fat, high in carbohydrate, or from intermediate 'balanced' diets." However, careful examination of their data shows that there was a decided wastage of energy in the growth metabolism of the rat on the high carbohydrate diet, for in this case 10 per cent more food was consumed than on either of the other diets during the period of comparable growth. Smith and Carey (1923) point out further that, "At whatever age they are compared, the high carbohydrate-fed rats consumed more calories than the standard diet rats growing at the same rate."

*Experimental Results.*—In Table II is illustrated the efficiency, *in growth*, of calories furnished, on the one hand, by a diet (Fat Diet I) practically devoid of carbohydrate and containing 86 per cent of fat calories, and, on the other, by a better balanced diet (Standard Diet V) consisting of 47 per cent of fat calories and 39 per cent of carbohydrate calories; in both rations, the ratio of salt and of protein calories to the total calories was the same.

In Table II care was taken, as far as possible, in making the comparisons of calorie utilization to select animals of the same initial weight and making the same gain in body weight in the same time. Obviously, this precaution is of great importance in ascribing value to results of calculations. It will be noticed that two ranges on the growth curve were chosen, *i.e.* gains of 63 and 93 gm. In the last column in Table II, *the average caloric intake per 100 gm. of live weight* is given. This value serves as an index of the efficiency of the particular diet on which the animal is grown. The higher this value, the less efficient is the diet in furnishing calories for the growth purposes.

TABLE II.  
*Utilization of Calories in Growth.*

Fat Diet I.			Standard Diet V.			
		Apportionment of calories.			Apportionment of calories.	
	<i>per cent</i>			<i>per cent</i>		
Casein.	25	Protein. 14.0	Casein.	18	Protein. 14.0	
Lard.	64	Fat. 86.0	Lard.	23	Fat. 47.0	
Cod liver oil.	5		Cod liver oil.	4		
Salt mix- ture.	6		Starch.	51		Carbo- hy- drate.
			Salt mix- ture.	4		
	100	100.0		100	100.0	

*Average Gain in Weight in Chosen Period = 93 Gm.*

Rat No.	Weight at start of chosen period.	Weight at end of chosen period.	Gain in weight.	Time to make gain.	Caloric intake for period.	Average* caloric intake per 100 gm. of live weight per day.
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Rats grown on Fat Diet I.

	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>days</i>		
60	66	157	91	66	2294	31
61	69	166	97	66	2279	29
62	65	152	87	66	2035	29
63	70	168	98	66	2390	30
64	70	161	91	66	2264	30
Average.....			93	66	2254	30

Rats grown on Standard Diet V.

368	61	153	92	56	1905	32
369	65	157	92	63	2077	30
370	66	161	95	56	2016	32
371	64	151	87	61	2212	34
372	70	166	96	61	2226	31
Average.....			93	60	2087	32

TABLE II—*Concluded.**Average gain in Weight in Chosen Period = 63 Gm.*

Rat No.	Weight at start of chosen period.	Weight at end of chosen period.	Gain in weight.	Time to make gain.	Caloric intake for period.	Average* caloric intake per 100 gm. of live weight per day.
Rats grown on Fat Diet I.						
362	51	111	60	47	1299	34
363	53	117	64	41	1241	36
364	52	113	61	49	1424	34
365	47	113	66	41	1140	35
366	53	115	62	41	1080	31
367	48	108	60	41	1184	37
Average.....			62	43	1228	35

Rats grown on Standard Diet V.

368	56	115	59	41	1299	37
369	49	112	63	41	1150	35
370	51	110	59	27	763	35
371	47	116	69	41	1193	36
372	53	120	67	41	1200	34
Average.....			63	38	1121	36

\* This value calculated by use of the following formula:

$$\frac{\text{Average daily caloric intake for period}}{\text{Mean weight of the rat during the period}} \times 100 = \text{Average caloric intake per 100 gm. of live weight per day.}$$

When the 93 gm. gain in weight is chosen, it is observed that both of the diets employed—Fat Diet I and Standard Diet V—were about equally efficient in promoting growth. Thus, the average values obtained were respectively 30 and 32. Employing a smaller range on the growth curve—63 gm.—and using a different set of rats on Fat Diet I, one obtains similar results, the average values being respectively 35 and 36. The average values for each group when treated statistically were shown to be mutually inclusive. *From these results, it appears, therefore, that rats receiving a maximum of calories in the diet in the form of fat can grow as efficiently*

as when a mixture of fat and carbohydrate calories is present in the ration. Smith and Carey (1923) arrived at the same conclusion.

Another interesting feature is demonstrated in Table II. It is seen that the *daily caloric intake per 100 gm. of live weight* diminishes as the length of time increases during which growth was observed. Thus, when a range of 63 gm. was selected, the average values were 35 and 36, while with a longer range—93 gm.—the resulting average values were much smaller—30 and 32. In calculating the data of Smith and Carey (1923) who employed the same diets but chose a much smaller range—19 to 21 gm.—average values of 37 to 39 and 42 were obtained respectively. These results bear out those of Macallum (1919) who found that the caloric requirements displayed a tendency to diminish with the increasing age of the rat.

### *III. Absorption of Fat in Rats on Various High Fat Diets.*

Although apparently no workers have attempted the study of the utilization of fat when preformed carbohydrate is almost entirely omitted from the food ration, various investigators have made similar studies under other dietary conditions. In investigations of this nature, human subjects as well as rats, dogs, and cats have been used.

Experiments on man, the dog, and the cat with a wide variety of fats have uniformly shown utilizations near 95 per cent (Langworthy (1923), Holmes (1926), Levites (1906), Pettenkofer and Voit (1873), Lyman (1917), Hill and Bloor (1922)). In connection with the present investigation, the work of Drummond (1919) is of interest. This investigator determined the utilization of fat in rats on a purified food ration (similar to Standard Diet V) containing 16 per cent of fat. The feces were collected for five successive periods of 7 days each. Almost complete absorption of fat resulted as was evidenced by the high utilization value of 97.3 per cent.

*Experimental Results.*—In the present investigation, since the animals in Groups A, D, and E (see Charts I and II) exhibited a growth rate slightly below normal it was important to ascertain how well the fat in the various high fat rations (Fat Diets I, II, and III) was utilized by the rats. Accordingly, the feces of the animals in the three groups were collected in five successive periods of 7 days each. This procedure was carried out after the animals

had been on the various diets for some time. The amount of fecal fatty material was then determined by ether extraction in a Soxhlet apparatus. Table III shows the utilization of fat on the various régimes.

TABLE III.  
*Absorption of Fat in Rats on Various High Fat Diets.*

Rat No.	Period (1). Fat absorption.	Period (2). Fat absorption.	Period (3). Fat absorption.	Period (4). Fat absorption.	Period (5). Fat absorption.
Group A (Fat Diet I).					
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
982	99.2	99.0	99.0	99.4*	98.8*
983	96.8	98.5	98.9	99.1*	98.4*
984	98.1	98.0	98.8	99.0*	98.3*
985	99.3	97.9	99.2	99.3†	97.7†
986	96.2	99.2	99.3	99.4†	97.5†
987		98.9	99.3		
Group D (Fat Diet II).					
988	99.0	99.0	99.2	98.8†	97.7†
989	98.9	99.0	99.4	99.2†	98.9†
990	98.8	98.5	98.8		
991	98.4	98.3	98.8		
992	98.8	99.1	99.3		
993	98.9	98.0	98.5		
Group E (Fat Diet III).					
994	99.2	99.0	99.0	99.3†	99.1†
995	98.8	98.9	99.2	98.7†	97.8†
996	98.5	98.5	99.3	98.9†	98.4†
997	97.5	97.9	99.3	98.9†	98.8†
998	94.3	98.4	98.7	97.7†	
999	98.4	98.8	99.2		

\* Change to Fat Diet II.

† Change to Standard Diet V.

‡ Change to Fat Diet IV.

It is at once evident that the fat in all the experimental diets was almost completely absorbed by the rats. The average value for the utilization of fat in all three groups was approximately 98 to 99 per cent. In Periods 4 and 5 when changes in the dietary

were made, this value did not change. Thus, in these periods, in Group A, when Fat Diet I was replaced by Fat Diet II, this value did not materially change. Again, substitution, in these periods, in Groups A, D, and E, with Fat Diet IV and Standard Diet V—diets containing greater amounts of preformed carbohydrate than fat—did not affect the values of fat absorption.

*The above results furnish convincing evidence that rats can utilize fat almost completely even when the fat content of the diet amounts to 86 per cent of the total calories ingested.*

#### SUMMARY.

1. Growth at a normal rate from 30 to 180 gm. in body weight can be induced in rats on a high fat ration practically devoid of preformed carbohydrate and containing 86 per cent of the total calories in the form of fat, provided the protein and salt requirements of the animal are fulfilled.

2. The livers of some of the rats that had been on the above high fat ration for a period of 83 days when examined microscopically did not contain fat droplets.

3. Comparisons were made of the efficiency, *in growth*, of calories furnished, on the one hand, by a high fat diet (practically devoid of preformed carbohydrate and containing 86 per cent of the calories in the form of fat) and on the other, by a better balanced ration (containing 39 per cent of calories derived from carbohydrate and 47 per cent of the calories in the form of fat). It appears that rats receiving a maximum of calories in the diet in the form of fat can grow as efficiently, measured by energy cost, as when a mixture of calories derived from both fat and carbohydrate is present in the ration.

4. Determination of fat utilization on the high fat diets here employed showed that the rat can utilize from 98 to 99 per cent of the ingested fat.

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## THE BEHAVIOR OF THE PROLAMINS IN MIXED SOLVENTS. II.

By D. B. DILL.\*

*(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)*

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A quantitative description of the solubility behavior of gliadin in mixtures of water with certain alcohols has been given recently by Dill and Alsberg (1). There now has been completed a similar study of zein, a companion protein. In addition to mixtures of water with methyl, with ethyl, and with *n*-propyl alcohol, mixtures of water with tertiary butyl alcohol have been employed as solvents. Also, observation has been made of the behavior of gliadin in mixtures of water with tertiary butyl alcohol.

The zein preparation used is the one of Professor Osborne used by Cohn, Berggren, and Hendry (2). The gliadin was prepared by the author according to the method of Dill and Alsberg (1). Their experimental procedure for determining turbidity temperatures was followed without modification.

It had been found that there is no well defined upper limit to the solubility of gliadin in certain alcohol-water mixtures and within certain temperature limits. The results of two preliminary experiments on zein, detailed in Table I, indicate that this prolamins may be characterized similarly. These results, together with a former similar experiment with gliadin, are illustrated in Fig. 1. In the indicated mixtures of alcohol and water, the solubility of gliadin and of zein increased rapidly with the temperature until a concentration of about 3 gm. per 100 gm. of solution was reached. At this point no further increase in temperature was required to dissolve a great deal more protein.

That this curious phenomenon is not explained by supersaturation was proved by observing both the appearance of turbidity on

\* National Research Fellow in Chemistry.



cooling and its disappearance on warming. In a given solution, turbidity appeared and disappeared at about the same temperature.

It is difficult on account of increasing viscosity to prepare in the usual manner zein solutions more concentrated than 25 per cent. However, by evaporation at 50°C. of concentrated solutions of zein or of gliadin in 70 per cent *n*-propyl alcohol, a more and more

TABLE I.  
*Turbidity Temperatures of Solutions of Zein in Alcohol-Water.*

Zein.	Temperature at which turbidity appeared on cooling from 50°.	Temperature at which turbidity disappeared on warming from 0°.
A. 70 per cent EtOH by volume in the solvent.		
gm. per 100 gm. solution	°C.	°C.
0.53	6	7
0.98	10	11
1.13	11	9.5
2.06	12	11
3.36	12	14
5.23	12	12
8.33	11	13
8.89	12	14.5
14.51	13	14
B. 58 per cent EtOH by volume in the solvent.		
0.20	19	19
0.75	30	31
1.66	30	32
3.33	34	32
4.74	33	34
12.15	33.5	34

concentrated solution is obtained without loss of apparent homogeneity. Finally, a perfectly transparent and dry film is obtained. The statement is justified, then, that there is no well defined upper limit to the solubility of gliadin or of zein in suitable alcohol-water mixtures within certain temperature limits. Therefore, the phenomenon may be described appropriately by the term peptization. The determination of the solubility of zein by Galeotti and Giampalmo (3), quoted by Seidell (4), was in reality a determination of

the relative rates of peptization in each of the various solvent mixtures used. There was no evidence presented that saturation was attained and we are safe in concluding that it was not attained.

Two series of solutions of zein in the various solvents were then prepared. One series contained approximately 4 per cent, and the

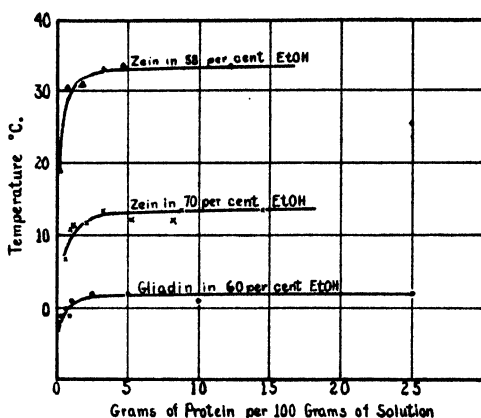


FIG. 1. Solubility of zein and of gliadin in certain EtOH-H<sub>2</sub>O mixtures.

TABLE II.

*Turbidity Temperatures of 4 Per Cent and 8 Per Cent Solutions of Zein in Methyl Alcohol-Water Solvents.*

Methyl alcohol.		Turbidity temperature.	
		4 per cent zein solution.	8 per cent zein solution.
vols. per cent	mol fraction	°C.	°C.
70	0.483	> 65	> 65
80	0.609	45	46
85	0.684	38	42
90	0.772	44	47
95	0.875	> 65	> 65

other 8 per cent zein by weight. The turbidity temperatures for zein in methyl alcohol-water mixtures are given in Table II; in ethyl alcohol-water, Table III; in *n*-propyl alcohol-water in Table IV. The turbidity temperatures for 4 per cent zein solutions and

TABLE III.

*Turbidity Temperatures of 4 Per Cent and 8 Per Cent Solutions of Zein in Ethyl Alcohol-Water Solvents.*

Ethyl alcohol.		Turbidity temperature.	
		4 per cent zein solution.	8 per cent zein solution.
vols. per cent	mol fraction	°C.	°C.
40	0.163	> 65	> 65
45	0.192	> 65	> 65
50	0.224	55	59
58	0.282	34	33
60	0.299	27	28
70	0.394	12	12
80	0.521	6	7
85	0.601	9	
86.2	0.624	14	15
90	0.700	32	21
95	0.826	> 65	> 65

TABLE IV.

*Turbidity Temperatures of 4 Per Cent and 8 Per Cent Solutions of Zein in n-Propyl Alcohol-Water Solvents.*

n-propyl alcohol.		Turbidity temperature.	
		4 per cent zein solution.	8 per cent zein solution.
vols. per cent	mol fraction	°C.	°C.
10	0.026	> 65	> 65
20	0.056	> 65	> 65
30	0.092	> 65	> 65
40	0.135	53	49
50	0.188	17	17
60	0.255	-5	-5
70	0.345	-15	-16
75	0.400	-17	
80	0.468	-9	-11
85	0.548	-7	
90	0.650	17	17
95	0.785	> 65	> 65

for 4 per cent gliadin solutions in tertiary butyl alcohol-water are shown in Table V.

In these tables, the concentrations of the three primary alcohols are shown both in terms of volumes per cent and mol fraction. Since the latter is the more fundamental unit, it is used in plotting the zein results which are shown graphically in Fig 2. The new gliadin results, together with the earlier observations, are illustrated graphically in Fig. 3. Here, too, the concentrations are in terms of mol fraction.

TABLE V.

*Turbidity Temperatures of 4 Per Cent Solutions of Zein and of Gliadin in Tertiary Butyl Alcohol-Water Solvents.*

Tertiary butyl alcohol.	Turbidity temperature.	
	4 per cent zein solution.	4 per cent gliadin solution.
<i>mol fraction</i>	<i>°C.</i>	<i>°C.</i>
0.018	> 65	46
0.037	> 65	24
0.071	> 65	12
0.102	26	23
0.133	8	> 65
0.160	7	> 65
0.188	-5	> 65
0.232	-6	> 65
0.274	6	> 65
0.330	23	> 65
0.362	> 65	> 65

The solubility behavior of nitro cotton is not unlike that of gliadin and of zein. It dissolves in many pairs of mixed solvents, even though it may be insoluble in one of the components. There is no well defined upper limit to its solubility. Evaporation of its solutions may yield a transparent film. A theory explaining its solubility behavior has been advanced by McBain, Harvey, and Smith (5). The significant portion of their theory is as follows:

"The fundamental facts operating in the behavior of solvents and nitro cotton is direct combination between solvent and suitable complementary chemical groups in the nitro cotton. The apparent viscosity of the solutions is almost entirely due to the presence of loose ramifying aggre-

gates of colloidal particles united by local and specific bonds of residual affinity of different kinds and degree. The best solvents are those which most effectively combine with these bonds . . . and dismember the aggregates. Hence, the best solvents yield solutions of the lowest ap-

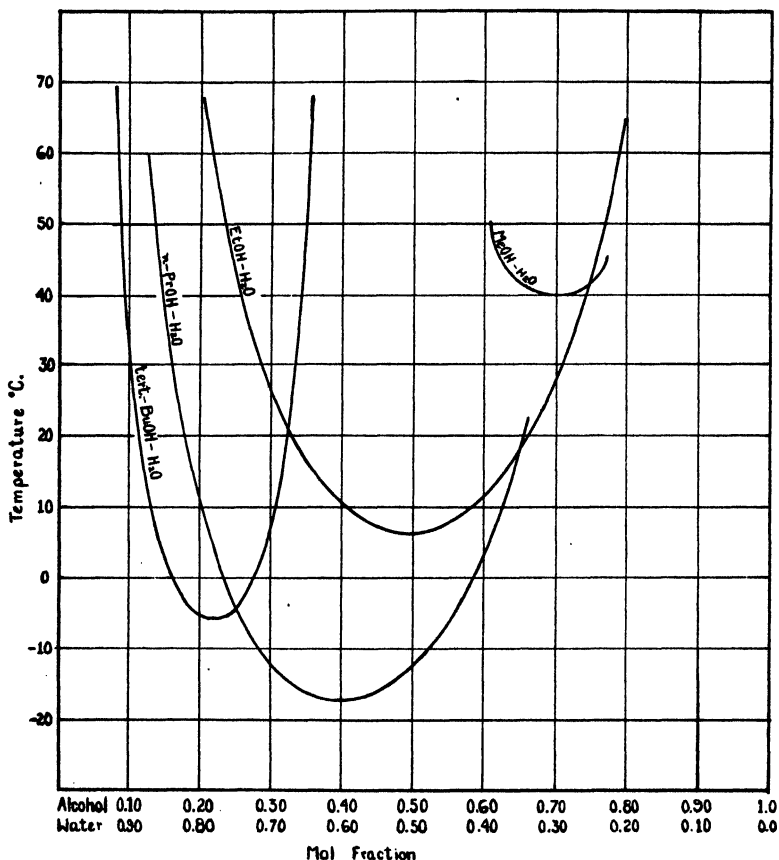


FIG. 2. Critical peptization temperatures of zein in MeOH-H<sub>2</sub>O, EtOH-H<sub>2</sub>O, *n*-PrOH-H<sub>2</sub>O, and tertiary BuOH-H<sub>2</sub>O.

parent viscosities and mixtures of solvents and 'non-solvents' affording a variety of suitable chemical groups are much more efficient than a single solvent."

If this theory is to be applied to prolamin solutions in mixed solvents, it must be in accord with the following observations.

1. For a given alcohol-water mixture, there is a "critical peptization temperature." Below this temperature, the solubility rapidly decreases. At, or above this temperature, there is no well defined upper limit to the solubility. It may be added as a corollary that the position and shape of the curves are independent of the protein concentration when this exceeds a minimum of 2 to 3 per cent.

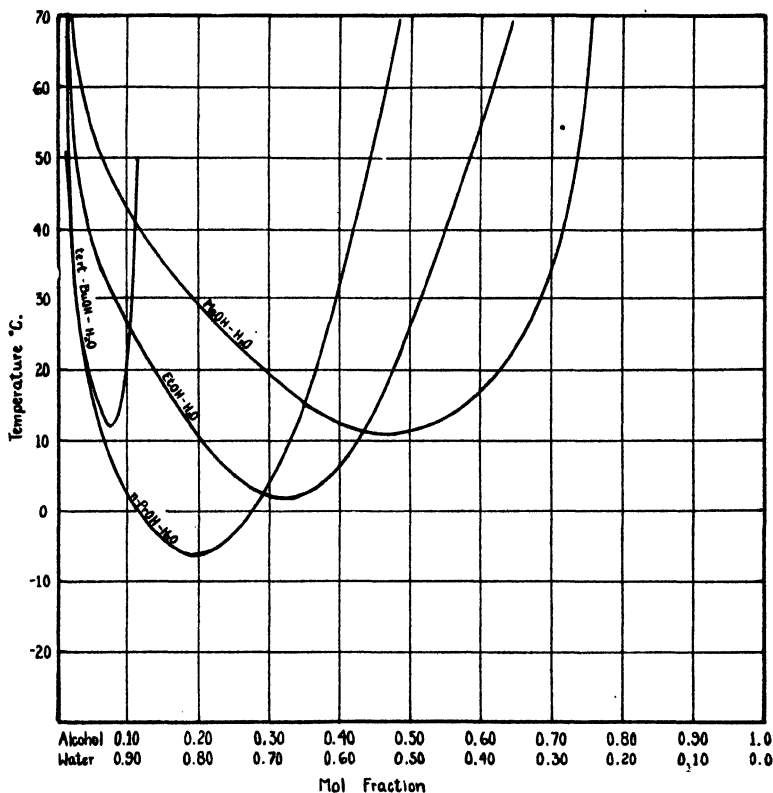


FIG. 3. Critical peptization temperatures of gliadin in MeOH-H<sub>2</sub>O, EtOH-H<sub>2</sub>O, n-PrOH-H<sub>2</sub>O, and tertiary BuOH-H<sub>2</sub>O.

2. In the case of the three primary alcohols, the relative position of the three curves for each protein bears a qualitative relationship to the molecular weights of the alcohols. The position of a given curve appears to be a function of the molecular weight of the alcohol.

3. For a given alcohol, the gliadin and zein curves are quite different, and it follows that the position and shape of the curve is a function of the character of the protein. There is a suggestive correlation between the properties of these two proteins and their behavior in these two mixed solvents. Cohn and his associates (2, 6) have shown that zein is one of the most inert of proteins. It has a very large molecule and it is not amphoteric inasmuch as it does not combine with acid. Gliadin is amphoteric, is less inert, and has a smaller molecule. The difference in the solubility behavior of these two proteins accords with these properties. In the case of the primary alcohols, zein is dissolved in a narrow range of methyl alcohol-water mixtures and in a very wide range of *n*-propyl alcohol and water, with ethyl alcohol and water occupying an intermediate position. A reciprocal relationship holds for gliadin in these mixed solvents. Of these three alcohols, methyl alcohol is most polar and *n*-propyl alcohol is least polar. Their dielectric constants are MeOH, 35; EtOH, 27; *n*-PrOH, 22. That of tertiary BuOH is 11.

There is a similar contrast in the behavior of these proteins in mixtures of water with the relatively non-polar tertiary butyl alcohol. At 50°, the alcohol concentration range for the gliadin solvent is from a mol fraction of 0.02 to one of 0.12, while the range for the zein solvent at this temperature is from 0.09 to 0.35.

The theory of McBain *et al.* accounts for all these phenomena except the sudden transition from turbidity to homogeneity, independently of protein concentration. The suggestion is advanced that this is a freezing point phenomenon; that a given alcohol-water mixture dissolves the protein with the formation of an alcohol-water-protein complex. Above the "critical peptization temperature," this complex is liquid and miscible in all proportions with this alcohol-water mixture. At, and below this temperature, it is solid and has definite and limited solubility.

#### SUMMARY.

A qualitative description has been given of the solubility behavior of zein in mixtures of water with methyl alcohol, with ethyl alcohol, and with *n*-propyl alcohol. The solubility behavior of both zein and gliadin in mixtures of water with tertiary butyl alcohol has been studied.

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## CHANGES IN THE BLOOD SUGAR OF THE COD, SCULPIN, AND POLLOCK DURING ASPHYXIA.

By MAUD L. MENTEN.

(From the Pathological Laboratories, University of Pittsburgh, Pittsburgh, and  
the United States Fisheries Station, Boothbay Harbor, Maine.)

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Wide variations in the dextrose content of blood, both under normal and pathologic conditions, in different fish have recently been reported (1-5) but without an adequate explanation of the causes underlying these differences, although asphyxia has been shown to be an important factor. Previous estimations have been made on single withdrawals of blood from individual fish. In the present paper, consecutive samples of blood obtained from the heart of the same fish during progressive asphyxia were analyzed.

In order to obtain blood by heart puncture a fairly large fish is necessary, so that the types mainly studied were the cod (*Gadus callarias*), pollock (*Pollachius virens*), and sculpin (*Myoxocephalus octodecimspinosus*). The latter measured between 12 and 15 inches. The cunner (*Tautogolabrus adspersus*), lump fish (*Cyclopterus lumpus*), flounder (*Pseudopleuronectes americanus*), and smooth dogfish (*Mustelus canis*) were also studied. All fish except the cunner and sculpin were obtained from a trap 6 miles from the station to which they were transported in tank or towing car. A few cod were obtained by hand line. The cunners were obtained by crab net and sculpin either with seine or fyke net. The fish were kept in aquaria adequately oxygenated with running sea water and fed on chopped clams, which are more readily digested than the small herring, smelts, squids, and mussels naturally obtained. Blood was obtained not only from fish kept in aquaria but also from cod and sculpin immediately on their removal from the sea by hand line or net.

Blood was withdrawn by syringe either from the heart, bulbous aortæ, or large vessels leading from the dorsal aorta, the positions

of which were defined by preliminary dissections. In the pollock and dogfish the heart was reached by inserting the needle on the ventral surface of the fish under the posterior edge of the shoulder girdle and extending it forwards and slightly downwards into the isthmus. In the cod and sculpin, where the bone covering the pericardium is more extensive, the heart was reached by insertion of the needle at the point of maximum pulsation on the lateral wall of the pericardium which had been exposed by drawing aside the operculum. Any appreciable bleeding within the pericardium was prevented by the rapid clotting of the blood. The cod, which succumbed readily to lack of oxygen, rarely survived longer than 1 hour after the first bleeding and could be bled usually 3, at most, 4 times. The sculpin, however, could be maintained in low concentrations of oxygen for 3 or 4 hours and could be bled easily 5 to 7 times, consecutively.

The treatment of the blood and estimation of dextrose was that recommended by Folin and Wu (6). The facility with which fish blood clotted necessitated the addition of 4 to 5 times as much potassium oxalate as is used with human blood. Furthermore, because of the low sugar content of normal blood in fish when 1 cc. of blood was used for analysis the standard adopted contained 5 instead of 10 mg. of dextrose per 100 cc. Results were checked by taking 3 cc. of blood and examining two samples consisting of 1 and 2 cc., respectively. To the 2 cc. sample were added 5 cc. of distilled water and 1.5 cc. each of 10 per cent sodium tungstate and  $\frac{2}{3}$  normal sulfuric acid, and the filtrate was compared against a colorimetric standard containing 0.1 mg. of dextrose per cc. Both samples gave practically the same value.

Asphyxial conditions were induced by placing the fish, immediately subsequent to withdrawal of the first sample of blood, in a pan containing about 5 gallons of water and allowing it to exhaust the oxygen supply by respiration.

The minimum sugar values, which may reach as low as 0.014 per cent, were observed in the sculpin, which had been deprived of food for several days. This is in agreement with figures reported by Simpson (4). The amount of food ingested and the length of time it remains in the intestinal tract undergoing digestion profoundly modify the reducing value of the blood. Alimentary hyperglycemia may persist for days. Eight unselected

TABLE I.  
*Variations in Blood Dextrose of Fish during Asphyxia Following Fasting and Feeding.*

	No. of fish from which average value was obtained.	Mg. of dextrose per 100 cc. of blood.							Remarks.
		Before asphyxia.	Time in hrs. after beginning of asphyxial conditions.						
			1	1	1½	2	2½	3	
<i>Myoxocephalus octodecimspinosus</i> . . . . .	4	16	24	32	41	54	63	Kept in aquarium a wk. without food. Previously fed and deprived of food 48 hrs. before experiment. Stomachs distended with food undergoing digestion. Taken with hand line. No food in stomach.	
“	4	23	42	76	167	200	208		
“	3	37	52	58	83	117	120		183
<i>Gadus callarias</i> . . . . .	7	35	43	50	56			Had been kept in aquarium and fed or taken from trap.	
“	7	115	130	154	226				
<i>Pollachius virens</i> . . . . .	1	30	46	51				Kept in aquarium without food several days.	
“	8	92	114	141	167			Taken from traps. Food in stomach.	
<i>Cyclopterus lumpus</i> . . . . .	1	34	39	42	50			Kept in aquarium several wks. No food ingested.	
<i>Mustelus canis</i> . . . . .	1	59	59	59	70	77		Kept in aquarium 2 wks.	

sculpins gave the following percentages of dextrose; namely, 0.029, 0.034, 0.04, 0.043, 0.05, 0.073, 0.088, and 0.1. Small fish in varying stages of digestion (and decomposition) were found in the stomachs of the last six.

The hyperglycemia, therefore, induced by asphyxia depends in large part upon the blood dextrose at the commencement of the asphyxial conditions and this in turn is largely governed by the quantity of previous foods as well as the time of intake previous to the withdrawal of blood. The relation of the rise in dextrose following asphyxia to the initial content of blood dextrose is shown by results presented in Table I.

The onset of hyperglycemia is not immediate but develops about 1 hour after the beginning of the asphyxial conditions. This probably is conditional upon the development of other deficiencies since little hyperglycemia develops when death is due to a rapidly developing asphyxia, such as occurs when the animal dies in the air, as previously noted by Denis (1).

In the cod, which is very susceptible to lack of oxygen, the normal glycemia lies between 30 and 35 mg. per 100 cc. of blood. During digestion this figure may rise to 200 mg. As in the sculpin the hyperglycemia occurring during asphyxia is proportional to the initial blood sugar. Blood taken from the cod immediately on its removal from the sea by hand line invariably showed a minimum dextrose content and no food in the intestinal tract, while blood withdrawn from the cod, taken from the trap, with a stomach distended with food showed a hyperglycemia. Only one pollock had a low blood sugar of 30 mg. per 100 cc. of blood and this presumably represented a normal value. Depriving pollock, which had been previously freely supplied with clams, of food for 48 hours did not materially lower the blood sugar. The hyperglycemia developing from asphyxiation is of the same degree as in the cod.

In eighteen cunners the average value of the blood dextrose was 0.034 per cent. As these fish had been fasted for several days, this probably represented a normal glycemia. A similar value, namely 0.038 and 0.051 per cent, was found for the two flounders and 0.034 per cent for one lump fish. The effects of asphyxia on glycemia in this lump fish are recorded in Table I. In the one dogfish studied a value of 0.058 per cent dextrose was obtained,

with only a slight increase resulting from asphyxia (Table I). This is slightly lower than the normal value of 0.065 per cent reported by Scott (2). It is regretted since Denis (1) observed a wide variation between the blood of elasmobranch and teleost fishes in relation to the nitrogen constituents that only one sample of elasmobranch fish was available for study.

Although among the fish studied there is a considerable variation in the normal blood sugar, ranging from 0.014 to 0.058 per cent, the values are appreciably lower than those found in the mammalian blood and probably give a fair estimate of the reducing substances present in poikilothermic blood. The nature of this reducing substance is not known. Scott reports a similar value; namely, 0.034 per cent for the frog.

The state of alimentation undoubtedly modifies profoundly reducing substances of blood of fishes both in their normal environment and under asphyxial conditions. It seems probable, as suggested by Hall and his coworkers, that factors other than asphyxia contribute to the rise of reducing substances occurring with decreased oxygen intake, because of the period of time required for asphyxial hyperglycemia to manifest itself.

#### CONCLUSIONS.

1. Normal glycemia in the sculpin lies between 0.014 and 0.02 per cent, and in the cod and pollock between 0.03 and 0.04 per cent. In the one dogfish studied the dextrose content of the blood was 0.059 per cent.

2. The rise of blood sugar occurring in asphyxia begins an appreciable time after the onset of the asphyxial conditions and its degree is dependent upon the initial glycemia. This latter condition is largely governed by the amount of food ingested and the time of ingestion.

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## HYPERGLYCEMIA AND HYPOGLYCEMIA PRODUCED BY WITTE'S PEPTONE.

BY MAUD L. MENTEN AND HELEN M. MANNING.

(From the Pathological Laboratories, University of Pittsburgh, Pittsburgh.)

(Received for publication, December 1, 1926.)

The attention of one of us (M. L. M.) was first directed to the possible rôle of Witte's peptone in increasing the reducing substances of the blood on noting that a more marked hyperglycemia was produced by injections of filtrates of *Bacillus paratyphosus* B which contained this substance than by those which were free from it. Two conflicting views concerning the action of peptone on blood sugar were found in the literature. Henderson and Underhill (1) observed only a hyperglycemia following intravenous injections of Witte's peptone into dogs and rabbits, and Kuriyama (2) confirmed this observation on rabbits. McGuigan and Ross (3) on the contrary obtained in dogs a hypoglycemia appearing in 2 to 4 hours with little or no intervening hyperglycemia. It is well known that a considerable variation may exist among the terminal digestion products present in Witte's peptone. Our experiments on the production of hyperglycemia with other substances of unknown composition containing hydrolytic products led us to believe that the discrepancy between the two views might be explained by the difference in the composition of the samples used. This belief was borne out by our results, outlined below, which tend to reconcile the opposing views already advanced.

Of the five samples of Witte's peptone used, three were kindly supplied us by the Department of Physiology of this school. Solutions varying between 0.5 and 1 per cent were made with distilled water without heating and were injected into the marginal ear vein of rabbits, which, unless otherwise stated, were deprived of food for 24 hours previous to injections. The amount injected varied between 0.2 and 1 gm. per kilo of body weight and the volume usually did not exceed 3 cc. The blood was obtained by puncture



TABLE I.  
Showing Changes in Mg. per 100 Cc. in the Dextrose Content of the Blood Following Intravenous Injections of Witte's Peptone.

Sample of peptone used.	Dose of pep- tone in gm. per kilo.	Immediately before injection.	Time in hrs. after injection.												Remarks.	
			1	2	3	4	5	6	24	48	96					
1	0.3	103	107	108	90	108	104	105	116	111	111	Repeated after 8 days.  Average of results obtained from three different rabbits.				
	0.4	103	110	108	147	168	155	115	128	128	110				115	
	0.6	113	143		166	204	138	154	103	105	116				111	110
	0.7	102	125	119	142	119	116	111	77	97	115					
	0.8	111	113		133	108	100	106								
2	0.3	113			132	128	118		154	114		Repeated after 24 hr. interval. Animal died during night.				
	0.5	114	111	111	133	114	108	108	105	117						
	0.7	91	107	107	125	109	105	87	82							
	0.7	112	116		105	89	71	70	67							
3	0.2	104			117	125	140	143		129	98	111	Rabbit fasted 4 days. Repeated after 24 hr. interval. Animal mori- bund, killed. Rabbit fasted 4 days. Repeated after 24 hr. interval. Animal died during night. Fasted 4 days previous to injection.			
	0.8	131	185		230	237	248	222	192							
	0.8	83			48											
•	0.8	130	297		300	333	311	274	175	145						
	0.8	133			100	100	92	80	73							
	0.9	114	253													

[illegible]

\* These three rabbits died within 5 minutes following the injection.

of the ear veins and clotting was prevented by addition of potassium oxalate crystals to the shed blood. The method of obtaining the filtrate and for the estimation of dextrose was that recommended by Folin and Wu (4). Three of the samples of peptone (Nos. 1, 2, and 3) gave a moderate degree of hyperglycemia without any hypoglycemia unless the injection was repeated within an interval of 24 hours. One, Sample 4, gave a definite hypoglycemia in which a preceding hyperglycemia was either present or lacking, and the fifth sample produced no appreciable change in blood dextrose. The results obtained with the first four samples are tabulated in Table I.

The rabbits which responded with a slight hyperglycemia showed few or no symptoms and recovered completely within a few hours. A second dose of such a sample caused a similar reaction if an interim of 24 hours or more was allowed for the recovery of the animal. Following the injection of a moderately potent peptone (Samples 2 and 3) the hyperglycemia was accompanied by increased respiration, tachycardia, and voiding of urine, with return to a normal condition in 5 to 6 hours. With this peptone the animal did not recover sufficiently within 24 hours to withstand a reinjection at the end of that period and responded with a fatal hypoglycemia in 4 to 20 hours. The most potent peptone (Sample 4) produced a hypoglycemia following the initial injection. Unfortunately, only a small amount of this sample was available. The development of a hypoglycemia either with or without a preliminary rise of blood sugar was characterized by a progressively diminishing blood pressure and considerable difficulty was experienced 2 or 3 hours after the injection in obtaining blood except by heart puncture. No glycosuria was observed in any of the animals. This was probably due to impermeability of the kidney following injury as microscopic examination showed parenchymatous degenerations especially in the ascending limb of the loop of Henle and the proximal convoluted tubule. Judging from the extensive degenerative changes in the hepatic cells the injurious action of the peptone was exerted primarily upon the liver. The susceptibility of this organ may have been enhanced in certain animals showing variations by the presence of spontaneous infections of the *Bacillus paratyphosus* B group, which rabbits normally carry (5).

The greatest degree of pathologic alteration occurs in those rabbits dying with a hypoglycemia and consists in extensive vacuolation and deposits of brown pigment in the hepatic cells. Frequently the reaction is so severe that many of the cells are completely destroyed. Whether this toxic action results from a filtering out of the peptone by the endothelium of the sinusoids and a subsequent transfer to the liver cell or is due to an indirect action on the liver resulting from a parasympathetic irritation or a combination of both is not yet established. The hyperglycemia thus finds its explanation in the deranged cytoplasm of the liver cells with a release of their stored glycogen. Why a rapid rise in blood sugar terminating in sudden death in a few minutes should result from the injection in certain animals is not clear, although these results parallel similar fatal hyperglycemias reported by Zeckwer and Goodell (6) in bacterial anaphylaxis. The intensity of the morphological lesions produced in the liver by two consecutive injections of peptone separated by an interval of 24 hours, together with the concomitant hypoglycemia, indicates strongly that the decrease in blood sugar is referable to this organ. It seems probable that the extreme hepatic derangement induced either by single injections of a potent peptone or by repeated injections of a moderately toxic peptone results in an exhaustion and non-functioning of the liver which is comparable to the condition observed by Mann and Magath (7) after complete hepatectomy. Both procedures are followed by a hypoglycemia of such degree that it is incompatible with life.

#### CONCLUSIONS.

Different samples of Witte's peptone, when injected intravenously in rabbits, vary widely in the manner in which they modify glycemia. The change in blood sugar ranges from a lack of appreciable alteration through varying degrees of hyperglycemia to a fatal hypoglycemia.

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## **STUDIES OF THE METABOLISM OF WOMEN.**

### **III. VARIATIONS IN THE LIPID CONTENT OF BLOOD IN RELATION TO THE MENSTRUAL CYCLE.**

BY RUTH OKEY AND RUTH E. BOYDEN.

*(From the Laboratory of Household Science, University of California,  
Berkeley.)*

(Received for publication, December 7, 1926.)

As the variations in the lipid content of the ovary corresponding to the different stages in its activity have become known, there has been much speculation concerning the relationships between the metabolism of the lipids and the functional activity of the generative organs in women. The obvious outward sign of this activity is menstruation. Unfortunately, however, it has always been very difficult to judge at all exactly the time intervals from the menstrual period at which these changes occur in any given woman.

If we are to accept the evidence recently offered by Zondek and Ascheim (1, 2), and by Watrin (3) in conjunction with that of the earlier workers whose conclusions are summarized by Corner (4), we may probably consider that the process of maturation of the Graafian follicle begins within a few days after the end of a menstrual period. The extrusion of the ovum normally takes place sometime between the 10th and the 16th day after the onset of menstruation. After the ovum has escaped, the thecal cells become hyperplastic and the corpus luteum develops. The follicular liquid and the immature corpus luteum contain very little uncombined lipid. But, if the ovum is not fertilized, the growth of the corpus luteum continues for a period of from 10 days to 2 weeks only. It then undergoes a rapid retrogression which involves extensive fatty degeneration with the accumulation of free cholesterol and glycerides. Menstruation follows within 48 hours of the beginning of this retrogression.

While recent studies by Allen, Doisy, and their coworkers (5-7) on an apparently lipid-soluble estrus-producing ovarian hor-

mone have led to the conclusion that this substance is not identical with any of the known lipids; nevertheless its close association with cholesterol and fatty acids might lead us to think that these substances may not be entirely without significant functional activity in the ovary.

Zondek (8) and Zondek and Ascheim (1) as well as Allen (9) report that the ovarian hormone which causes estrus in spayed mice and rats is present in the human being not only in the mature follicles and the follicular liquid, but also in the non-pregnant corpus luteum up to the time of the beginning of its retrogression, and in the corpus luteum of the early stages of pregnancy. It is, however, absent from the degenerative corpus luteum menstruationis and from immature and atretic follicles.

Whether we are to consider, with Zondek (8), that this estrus-producing hormone is the only one elaborated by the ovary and is entirely responsible for the changes in the genital tract characteristic of ovulation and menstruation, or with Papanicolaou (10) that this is only one of three ovarian hormones, nevertheless the fact that the time of the disappearance of this hormone corresponds with that of largest accumulation of uncombined lipids in the ovary is suggestive.

It would seem that, whether we regard the lipids of the corpus luteum as in themselves functionally active, or whether we consider their accumulation as symbolic of cessation of hormonal activity, we might logically expect some variation in circulating lipids associated with the accumulation in and release of these substances from the generative tract. This is further indicated by the work of Ascheim (11) and of Mikulicz-Rodeki (12), who found extensive changes in the lipid content of the uterine tissues at various stages of menstrual activity. Since lecithin is known to be hemolytic in its properties and cholesterol antihemolytic, the cholesterol-lecithin ratio in the uterine mucosa associated with uterine bleeding might seem to have some significance.

Moreover, we have considerable evidence that cholesterol has a definite function in neutralizing bacterial toxins. Leopold and Seisser (13) believe that it protects the sex glands from toxins and that lowered blood cholesterol may be concerned in follicular degeneration. Denis (14) considers that hypocholesterolemia is associated with greatly lowered vitality, while convalescence

from acute infectious disease is characterized by an increase in blood cholesterol content.

However this may be, clinical experience indicates that there is a tendency to menstrual "flare-ups" in women with chronic infections and that possibly it may not be entirely illogical to connect these with certain menstrual variations in lipid metabolism, possibly with variations in lipid ratios in the blood.

Strikingly few studies of the blood lipid content in relation to the menstrual cycle have been reported. Moynihan (15) gives a type curve for blood cholesterol in women which shows a high value followed by a fall immediately before menstruation. The lowest point is reached toward the end of the menstrual period, and a somewhat higher level is indicated after the period. This is followed within 2 days by a fall to the normal level, which is maintained for 2 weeks. The work upon which his curves are based is attributed to Dr. Cecilia Shiskin and does not seem to have been published in full.

The most extensive investigation of blood cholesterol levels in relation to the menstrual cycle appears to have been that of Gonalous (16), which is reviewed by Chauffard (17). His curves are based on determinations of blood cholesterol levels made every 3rd day by the method of Grigaut. They would seem to indicate that there is a high value to be expected preceding and during the menstrual period, followed by a rather sharp drop to a normal level. The curves as given by Chauffard are, however, not quite of the type given by Moynihan. The work of Gonalous was unfortunately done largely on convalescent hospital cases, and the data do not seem to have been reported in full. It is therefore somewhat difficult to evaluate his results. Chauffard attributes to this periodic hypercholesterolemia the more frequent occurrence of gall stones in women than in men.

Numerous observations (Harding (18)) have indicated that there is an increased level of blood fat, lecithin, and cholesterol during pregnancy. Bacmeister and Havers (19), as well as Pribram (20, 21), attribute the increased blood cholesterol level to a decreased rate of elimination through the bile rather than to an increased rate of production. Various workers have attempted to explain the function of the cholesterol as a protective one, destined to neutralize toxins in the blood of the mother. Others (22) consider the increase in blood lipids a preparation for lactation.



While it is not within the scope of the present paper to deal with the general physiology of the regulation of the level of the various lipids in the blood stream, it seems necessary to mention certain outstanding findings concerning the factors which influence this level in order to consider intelligently data of the type we have to offer. It should perhaps be stated that the best general review of the subject is that by Bloor (23). Other articles which may be mentioned are those of Gardner and Fox (24) and of Knudson (25).

Bloor (26) found that during fat assimilation in normal dogs (1) total fatty acids increased in plasma and corpuscles, (2) lecithin increased greatly in the corpuscles and only slightly in the plasma, (3) no uniform change took place in the quantity of cholesterol, (4) there was a fairly constant relationship between total fatty acids and lecithin of blood and corpuscles. He considers that the corpuscles take up the fatty acid and transform it into lecithin, which he regards as an intermediary stage in the metabolism of fat. He suggests that the comparatively constant ratios which exist between the cholesterol and lecithin in normal blood are possibly indicative of some part played by cholesterol in the intermediary metabolism of fats. Myers (27) in his summary of the pathological variations to be expected in fasting values for blood lipids states that the most characteristic change is an increase in total fatty acids and fat, and a decrease in the plasma lecithin, probably indicating a diminished fat metabolism. In certain anemias a low cholesterol value is associated with a high blood fat, but as a rule when there is a general lipemia there is also a hypercholesterolemia.

The question of the relationship of absorption and synthesis of cholesterol to the level of this substance in the blood does not seem to be satisfactorily solved. Nor is the story of the regulation of its excretion through the bile entirely clear. Much stress is consequently placed in the clinical literature on the function of certain organs which contain cholesterol, *i.e.* adrenals, gonads, liver, etc., in their relation to the level of cholesterol in the blood and to cholesterol excretion. Hence, until more of the actual mechanisms involved in the metabolism of cholesterol become known, it would seem that we may be justified in considering that it may possibly, aside from the indefinite rôle which it has in fat metabolism and as an intermediary substance capable of taking

on antirachitic activity when exposed to light of short wave-length, be of importance because of its antihemolytic and antitoxic propensities. It would seem also that the regulation of the level of cholesterol in the fasting blood is in some way related to the activities of certain glands of internal secretion, notably the ovaries, adrenals, and thyroids.

#### EXPERIMENTAL.

The investigation reported here was undertaken in the hope of ascertaining whether or not there were any consistent cyclic variations in the fasting blood lipid ratios in women, and, if so, whether these variations took place at any consistently similar time intervals from the onset of menstruation.

#### *Subjects.*

The subjects were all students, chiefly seniors and graduates in the nutrition and related departments. A few of these were women who served also as subjects for investigations previously reported (28, 29), and with one exception (Ef.) all of them were rated as normal on the basis of University infirmary records at the beginning of the experiments. We were, however, unusually unfortunate in this particular group of subjects in the number of slight colds which developed during the period of observation. Also, our own questioning as to physical records, which we have always purposely delayed until we have felt sufficiently well acquainted with our subjects that we could expect frank answers, revealed in this group likewise a certain number of histories of menstrual abnormality. Since these were, as a rule, cases of past rather than present difficulty, and since there seemed to be a definite correlation between the history and the observations, it has appeared to us as best to include this data in our report, including also a somewhat fuller record of history than we have given space to in our accounts of similar experiments.

#### *Methods.*

The blood samples were taken before breakfast in the morning from the arm veins. A minimum amount of oxalate was used to prevent clotting. The blood was measured, dropped immediately

into a mixture of alcohol-ether, and the lipid extraction made according to the method of Bloor, Pelkan, and Allen (30) and Bloor (31). The analyses of the extract were completed at leisure by the methods given in the references cited above. Figures reported are averages of duplicate determinations which checked within the limits of accuracy to be expected when colorimetric and nephelometric methods are used.

It was found impractical to take large enough samples to determine the lipids in plasma and corpuscles separately, hence the figures given represent whole blood. Most of the analytical work recorded in Tables I and II was done by the junior author.

#### DISCUSSION.

The data from our determinations of before breakfast levels of blood lecithin, cholesterol, and total fatty acid in two subjects on constant diets are recorded in Table I. Table II shows corresponding before breakfast blood lipid levels in a larger number of women on uncontrolled diets, and Fig. 1 gives a record of more frequent determinations of before breakfast blood cholesterol levels in five additional subjects.

It will be seen from inspection of these tables and figure that the most consistent cyclic variation observed was the drop in the level of blood cholesterol which occurred almost invariably during or within a few days of the menstrual period. The lowest levels observed averaged 70 per cent of the observed average value for each individual, the lowest observed value being 46 per cent.

Preceding and following the menstrual drop in blood cholesterol level we have almost always found at least one value higher than the average level for the individual. Our highest observed values averaged 124 per cent of the averages of all observed values for the individuals concerned (Table III), the highest observed value being 157 per cent. Hence the observed variations within a week of the onset of menstruation amounted to 54 per cent of the average blood cholesterol values. The distribution of these variations is indicated in Table IV. In 50 per cent of our cases the lowest value was observed within 24 hours of the time of the onset of menstruation, in 73 per cent of the observed cases, within 48 hours of this time, while 84 per cent showed the drop within 3 days and 96 per cent within 4 days of the onset of the menstrual flow.

TABLE I.  
Blood Lipid Data, Group I.

Date.	Date in relation to menstruation.*	Cholesterol.	Fatty acid.	Lecithin as $\text{H}_2\text{PO}_4 \times 8$ .	Ratios.			Remarks.
					Fatty acid to cholesterol.	Lecithin to cholesterol.	Fatty acid to lecithin.	
Subject F., female, 31 years, height 67.4 inches, weight 65 kilos; unmarried.								
1924		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
Jan. 17	-6	189	270	264	1.43	1.39	1.02	Diet Jan. 18-Feb. 6:
" 20	-3	182	436	292	2.39	1.60	1.49	Egg 335 gm., cottage
" 23	0	172	320	244	1.86	1.42	1.31	cheese 150 gm., whole
" 25	+2	108	540	264	5.00	2.44	2.04	wheat bread 220 gm.,
" 26	+3	112	572	249	5.10	2.22	2.29	pecans 50 gm., cream
" 28	+5	122	492	286	4.03	2.34	1.72	43 gm., orange 100 gm.,
" 30	+7	130	480	247	3.69	1.90	1.94	apple 140 gm., lettuce
Feb. 2	+10	127	357	245	2.81	1.92	1.45	200 gm., postum 7 gm.,
" 4	+12	152	357	250	2.34	1.64	1.43	butter 35 gm., sugar 25
" 6	+14	196	305	220	1.55	1.12	1.38	gm. Day's intake ap- proximately: carbohy- drates 178 gm., fat 116 gm., nitrogen 16.1 gm., calories 2164.
Feb. 8	+16	222	354	261	1.59	1.17	1.35	Diet Feb. 8-Mar. 21: Po-
" 13	+21	169	424	266	2.50	1.57	1.59	tato 500 gm., white
" 18	+26	182	416	231	2.28	1.26	1.80	bread 200 gm., milk 200
" 19	0	137	508	252	3.70	1.83	2.01	gm., lettuce 200 gm.,
" 21	+2	112	556	268	4.96	2.39	2.07	apple 150 gm., orange
" 23	+4	123	400	278	3.25	2.26	1.43	200 gm., butter 60 gm.,
" 27	+8	250	460	254	1.84	1.01	1.81	sugar 60 gm. Day's
Mar. 3	+13	179	381	254	2.13	1.42	1.50	intake approximately:
" 6	+16	102	381	264	3.73	2.58	1.44	carbohydrate 318 gm.,
" 8	+18	132	472	261	3.58	1.97	1.80	fat 64 gm., nitrogen 5.2
" 12	0	196	404	261	2.06	1.33	1.54	gm., calories 2030.
" 13	+1	78	336	242	4.30	3.10	1.38	
" 15	+3	143	332	226	2.32	1.58	1.46	
" 17	+5	169	508	249	3.00	1.47	2.04	
" 21	+9	204	456	274	2.23	1.34	1.66	

The time intervals at which the high values preceded and followed the low ones were by no means uniform, varying from 1 to 14 days. In approximately half our cases the highest observed

value came after the actual beginning of menstrual bleeding and was followed by a rather sharp fall to the lowest observed level. In three cases, on the other hand, the duration of the fall in chole-

TABLE I—Continued.

Date.	Date in relation to menstruation.*	Cholesterol.	Fatty acid.	Lecithin as $\text{H}_2\text{PO}_4 \times 8$ .	Ratios.			Remarks.
					Fatty acid to cholesterol.	Lecithin to cholesterol.	Fatty acid to lecithin.	

Subject F.—Continued.

1924		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
Sept. 15	0	154	408	315	2.64	2.04	1.29	Diet Sept. 16–Nov. 8: Egg
" 16	+1	154	400	300	2.59	1.94	1.33	92 gm., milk 578 gm.,
" 18	+3	189	400	294	2.11	1.55	1.36	bread 250 gm., potato
" 22	+7	192	372	288	1.93	1.50	1.29	250 gm., tomato 219 gm.,
" 25	+10	222	370	288	1.66	1.29	1.28	apple 175 gm., grapes
" 29	+14	111	318	294	2.86	2.64	1.08	250 gm., orange 175 gm.,
Oct. 3	+18	127	326	291	2.56	2.29	1.33	lettuce 60 gm., butter
" 6	0	113	374	300	3.30	2.65	1.24	60 gm. Day's intake
" 9	+3	90	364	297	4.04	3.30	1.22	approximately: carbo-
" 10	+4	119	342	279	2.87	2.34	1.22	hydrate 310 gm., fat 83
" 11	+5	214	348	282	1.62	1.31	1.23	gm., nitrogen 10.9 gm.,
								calories 2252.
" 13	+7	250	354	288	1.41	1.15	1.22	Note: This subject suf-
" 16	+10	250	394	276	1.57	1.10	1.43	fered from several colds
" 18	+12	250	379	294	1.51	1.17	1.28	during the period of the
" 21	+15	193	370	300	1.91	1.55	1.23	experiment, one rather
" 25	+19	151	354	291	2.34	1.92	1.21	severe one Sept. 28–
" 27	+21	205	379	294	1.84	1.43	1.28	Oct. 6.
" 28	+22	202	392	267	1.94	1.32	1.46	
" 29	+23	300	377	294	1.25	0.98	1.28	
" 31	+25	272	364	273	1.33	1.00	1.33	
Nov. 1	0	193	404	288	2.09	1.49	1.40	
" 3	+2	300	370	297	1.23	0.99	1.24	
" 5	+4	202	408	282	2.01	1.39	1.44	
" 8	+7	189	408	285	2.15	1.50	1.43	

terol level was at least 6 days, in six cases it exceeded 5 days, and in nine cases 4 days. The average duration of the postmenstrual rise in blood cholesterol was slightly greater, but equally variable.

TABLE I—Concluded.

Date.	Date in relation to menstruation.*	Cholesterol.	Fatty acid.	Lecithin as $H_2PO_4 \times 8$ .	Ratios.			Remarks.
					Fatty acid to cholesterol.	Lecithin to cholesterol.	Fatty acid to lecithin.	
Subject C., female, 24 years, height 64 inches, average weight 67 kilos; unmarried.								
1924		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
Sept. 5	-6	111	412	300	3.71	2.70	1.37	Diet Sept. 5-26: Grapes 276 gm., egg 67 gm., whole wheat bread 203 gm., potato 280 gm., butter 104 gm., sugar 50 gm., milk 578 gm., orange 194 gm., lettuce 60 gm., pineapple 60 gm., cottage cheese 90 gm., cocoa 50 gm., tomato 219 gm. Day's intake approximately: carbohydrate 344 gm., fat 115 gm., nitrogen 14.5 gm., calories 2814.
" 8	-3	129	408	300	3.16	2.32	1.36	
" 11	0	125	400	285	3.20	2.28	1.40	
" 12	+1	112	400	288	3.57	2.57	1.38	
" 13	+2	172	377	294	2.19	1.70	1.28	
" 15	+4	200	353	294	1.76	1.47	1.20	
" 18	+7	192	363	297	1.89	1.54	1.22	
" 22	+11	182	363	285	1.99	1.56	1.27	
Sept. 25	+14	233	370	276	1.58	1.18	1.34	Sept. 25-Oct. 3: One egg substituted for cottage cheese. Fat intake increased to 122 gm. Cold; intestinal upset; diet discontinued Oct. 2.
" 29	+18	133	364	294	2.73	2.21	1.23	
Oct. 3	+22	150	374	294	2.49	1.96	1.27	
Oct. 30	-5	150	392	291	2.61	1.94	1.34	Diet as for previous period. Pineapple omitted, whole wheat bread changed to white bread, 276 gm. apple substituted for grapes. Total fat intake 117 gm.
Nov. 3	-2	158	394	288	2.49	1.82	1.37	
" 5	0	238	400	282	1.68	1.18	1.41	
" 6	+1	245	384	285	1.56	1.16	1.34	
" 7	+2	226	384	285	1.69	1.26	1.34	
" 8	+3	254	386	288	1.51	1.13	1.34	
" 10	+5	250	363	288	1.45	1.15	1.26	

\* Days preceding the onset of menstruation are indicated as (-) and days following the beginning of a period as (+).

Data taken during a menstrual period are printed in bold faced type.

TABLE II  
B) Lipid Data,

Subject.	Date.	Date in relation to menstruation.*	Cholesterol.		Fatty acid. mg. per 100 cc.	Lecithin as $H_2PO_4 \times 8$ mg. per 100 cc.	Ratios.			Remarks.
			mg. per 100 cc.	mg. per 100 cc.			Fatty acid to cholesterol.	Lecithin to cholesterol.	Fatty acid to lecithin.	
Subject N. Age 29 yrs., height 65 in., weight 62 kilos; mar- ried.	1924									
	Mar.	5	-12	112	339	266	3.02	2.37	1.27	Diet unweighed, but quite constant. Sub-
	"	13	-4	118	380	233	3.22	1.97	1.63	ject in very good physical condition
	"	18	+1	80	396	254	4.95	3.18	1.55	throughout experiment. History negative.
	"	19	+2	100	370	237	3.70	2.37	1.56	
	"	21	+4	111	292	283	2.63	2.55	1.03	
	"	24	+7	141	404	228	2.86	1.61	1.77	
Subject Nk. Age 21 yrs., height 67 in., weight 71 kilos; unmar- ried.	Feb.	7	+1	90	357	218	3.96	2.42	1.63	Diet unweighed, not greatly varied except
	"	9	+3	161	348	242	2.16	1.50	1.43	that subject ate a great deal of candy.
	"	11	+5	164	412	228	2.51	1.39	1.81	Usual history cramps 4 to 6 hrs. at begin-
	"	13	+7	149	363	254	2.43	1.70	1.42	ning of a menstrual period. History of a
	"	20	+14	156	416	276	2.67	1.76	1.50	bad cold Feb. 23-28.
	"	27	+21	89	366	278	4.10	3.12	1.31	
	Mar.	3	+26	132	392	266	2.96	2.01	1.47	
	"	6	0	105	363	266	3.46	2.53	1.36	
	"	8	+2	94	392	259	4.17	2.75	1.51	

Subject Fe. Age 19 yrs., height 65 in., weight 60 kilos.	Aug. 27	-14	130	400	287	3.07	2.20	1.39	Diet unweighed. History very nearly normal; menstrual periods usually 33 to 35 days apart.
	Sept. 1	-9	156	400	288	2.56	1.84	1.38	
	" 5	-4	143	397	282	2.77	1.97	1.40	
	" 10	0	172	400	279	2.32	1.62	1.43	
	" 11	+1	201	389	270	1.93	1.34	1.44	
	" 13	+3	130	400	279	3.07	2.14	1.43	
	" 16	+6	103	400	270	3.88	2.62	1.48	
	" 23	+13	149	384	297	2.57	1.99	1.29	
	Oct. 7	+27	185	370	294	2.00	1.58	1.25	
	" 13	+33	156	356	300	2.28	1.92	1.18	
	" 16	0	125	382	282	3.05	2.25	1.35	
	" 21	+5	150	379	261	2.52	1.74	1.45	
	" 28	+15	161	379	282	2.35	1.75	1.34	
Subject Nd. Age 31 yrs., height 65.3 in., weight 60 kilos.	Feb. 8	-8	172	396	245	2.30	1.42	1.61	History (10 yrs. previous to time of experiment) of menstrual difficulties diagnosed as due to retrodisplacement of the uterus, treated mechanically. Menstrual periods now normal.
	" 15	-1	164	644	269	3.92	1.64	2.39	
	" 16	0	100	480	268	4.80	2.58	1.86	
	" 18	+2	179	364	254	2.03	1.42	1.43	
	" 20	+4	141	464	254	3.29	1.80	1.82	
	" 27	+11	154	528	247	3.42	1.60	2.14	
	Mar. 12	+25	189	400	212	2.11	1.12	1.88	
	Feb. 11	-10	135	396	264	2.93	1.95	1.50	
	" 18	-4	115	520	221	4.52	1.92	2.35	
	" 21	0	101	420	293	4.15	2.90	1.43	
	" 25	+4	128	456	300	3.56	2.34	1.52	
	Mar. 5	+11	127	370	264	2.91	2.08	1.40	
	" 12	+18	135	500	276	3.70	2.04	1.81	
Subject Mb. Age 22 yrs., height 67 in., weight 63 kilos.	Feb. 11	-10	135	396	264	2.93	1.95	1.50	History negative. Interval between menstrual periods shorter than usual.
	" 18	-4	115	520	221	4.52	1.92	2.35	
	" 21	0	101	420	293	4.15	2.90	1.43	
	" 25	+4	128	456	300	3.56	2.34	1.52	
	Mar. 5	+11	127	370	264	2.91	2.08	1.40	



1-06

Subject.	Date.	Date in relation to menstruation.*	Cholesterol. mg. per 100 cc.	Fatty acid. mg. per 100 cc.	Lecithin as $H_2PO_4 \times 8$ . mg. per 100 cc.	Ratios.			Remarks.
						Fatty acid to cholesterol.	Lecithin to cholesterol.	Fatty acid to lecithin.	
Subject Mb. —Continued.	1924								
	Mar. 17	0	125	412	292	3.29	2.34	1.41	
	" 19	+2	115	338	268	2.94	2.24	1.31	
	" 21	+4	110	388	266	3.52	2.42	1.45	
Subject Td. Age 22 yrs., height 65 in., weight 52 kilos.	" 24	+7	145	500	274	3.44	1.88	1.82	
	Feb. 7	-24	108	370	202	3.43	1.87	1.83	History of irregular menstruation; never painful; otherwise normal.
	" 13	-18	139	396	252	2.84	1.81	1.57	
	" 20	-11	133	426	283	3.20	2.12	1.50	
Subject Qv. Age 23 yrs., height 64 in., weight 62 kilos.	Mar. 3	+1	149	392	228	2.63	1.53	1.71	
	" 5	+3	73	444	249	6.08	3.41	1.78	
	Feb. 9	+2	83	404	226	4.86	2.72	1.78	History negative. Diet somewhat varied, especially large consumption of candy. Habits not very regular, hard to control.
	" 11	+4	175	332	242	1.89	1.38	1.37	
	" 13	+6	164	436	245	2.65	1.49	1.69	
	" 20	+13	161	367	293	2.28	1.81	1.25	
	Mar. 5	0	159	305	286	1.91	1.79	1.06	
	" 12	+7	167	357	247	2.13	1.48	1.44	

Subject Y. Age 22 yrs., height 63 in., weight 50 kilos.	Feb. 18 " 21 " 23 " 25 Mar. 3	-3 0 +2 +4 +11	127 <b>96</b> <b>200</b> 169 133	512 <b>436</b> <b>528</b> 307 320	283 <b>262</b> <b>307</b> 245 233	4.03 <b>4.53</b> <b>2.64</b> 1.82 2.40	2.22 <b>2.72</b> <b>1.53</b> 1.44 1.75	1.80 <b>1.66</b> <b>1.71</b> 1.25 1.37	Menstrual history negative. Subject asth- matic. Diet tended to be acidic.
Subject Ef. Age 24 yrs., height 67 in., weight 80 kilos (approx- imately).	Jan. 28 Feb. 1 " 4 " 8 " 15	-3 +1 +4 +8 +15	106 <b>179</b> 164 167 192	332 <b>396</b> 381 396 492	278 <b>261</b> 266 261 293	3.13 <b>2.21</b> 2.32 2.37 2.56	2.62 <b>1.46</b> 1.62 1.56 1.52	1.19 <b>1.51</b> 1.43 1.51 1.68	Subject nephritic; supposedly on strict diet, poorly adhered to. Non-protein N 60-65. Blood pressure 170/120.

\* Days before a menstrual period are indicated as (-) and following the onset of menstruation (+).  
Data taken during a menstrual period are printed in bold faced type.

It is obviously unfair, in considering values observed at such infrequent intervals as those here reported, to assume that the low values observed represented the lowest levels reached, or to take

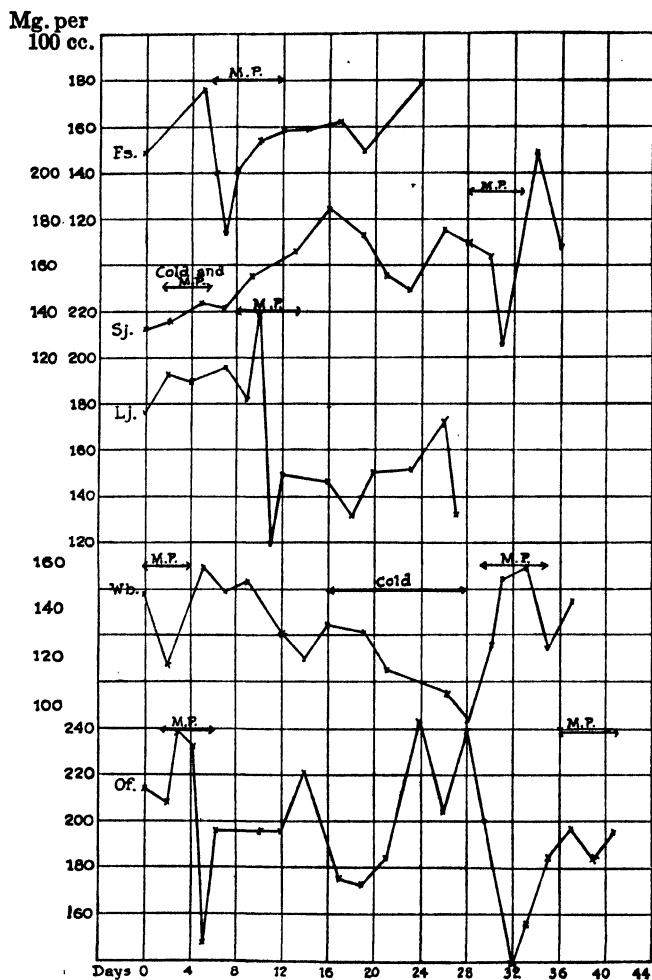


FIG. 1. Blood cholesterol curves, Group III.

observations covering only twenty-six periods in sixteen subjects as entirely representative of all the changes which occur in the blood lipid levels of normal women. However, the fact that

TABLE III.  
Summary. Blood Cholesterol Data.

	mg.	per cent
Average of all observed values.....	149	100
“ “ highest values observed within 7 days of menstrual period.....	185	124
Average of all lowest values observed within 7 days of menstrual period.....	98	70
Average of extent of largest observed menstrual variations (difference between high and low values for individual).....	84	54
Average of extent of largest observed intermenstrual variations (difference between high and low values for individual).....	41	28
Average of extent of largest intermenstrual variations, excluding cases where colds were complicating factors..	27	18

TABLE IV.  
Distribution of High and Low Blood Cholesterol Values for Each Period Studied.  
Percentage of Total Periods Studied Falling within Given Limits of Deviation.

Per cent of total period.	Lowest menstrual value.	Highest menstrual value.	Extent of largest observed variation.	
			Menstrual.	Intermenstrual.
Under 10				10
10-20				25
20-30			7.7	25
30-40			11.4	
40-50	7.7		11.4	20
50-60	7.7		34.6	10
60-70	30.7		15.3	
70-80	30.7		11.4	
80-90	19.2		7.7	
90-100	3.8			
100-120		3.8		
110-120		38.4		
120-130		30.7		
130-140		15.4		
140-150		3.8		
Over 150		7.7		

these changes in cholesterol level were so consistently observed when the opportunities for missing them were so ample would seem to point to some definite connection between the blood cholesterol and the menstrual phenomena.

When we attempt to correlate the time intervals at which the cholesterol variations are observed with the onset of menstruation, however, we find too great a lack of uniformity to draw definite conclusions. If, as would appear most probable from the histological observations on composition of corpus luteum, the premenstrual rise in blood cholesterol takes place as the corpus luteum nears maturity and the fall begins with the period of retrogression, we might perhaps assume that the accumulation of free lipid, especially cholesterol, in the corpus luteum takes place at the expense of circulating cholesterol, and that, as the retrogression proceeds, the cholesterol is gradually released. This would, however, be difficult to correlate with the short time intervals, sometimes less than 48 hours, occasionally observed between the premenstrual and postmenstrual peaks of the cholesterol curves. It should, however, be pointed out that the best histological evidence we have indicates that there are individual peculiarities in the time of formation and retrogression of the corpus luteum. Possible time relations between premenstrual lipid accumulation in the uterine mucosa and variations in blood lipid levels are also hard to establish in view of the irregularities in the intervals between the onset of uterine bleeding and the low point in the cholesterol curves.

Chauffard (17) places emphasis on the irregularities in blood cholesterol level as affecting cholesterol excretion through the bile and possibly accounting for the larger incidence of gall stones in women than in men. But he makes little or no attempt to account for the variation in blood cholesterol level other than to attribute it to general alterations in the activities of certain glands of internal secretion. Indeed, it would seem that any attempt to find the causal factors concerned in this variation must be based upon a more complete understanding of the general problems of lipid metabolism than is at present to be had.

The observed changes in blood fat and lecithin can hardly be said to bear a consistent relationship to any period of the monthly cycle in women. Nevertheless, the fact that a period of men-

strual hypocholesterolemia was more frequently characterized by an increase in blood fat than otherwise and that the lecithin level did not decrease with that of blood cholesterol would also appear to have some significance.

This becomes the more striking when we consider Bloor's suggestion (23), apparently based largely on observation of the smallness of normal variations in blood lipid ratios, that cholesterol in the form of its esters plays a definite part in fat metabolism. Myers (27) has stressed the value of blood cholesterol determinations as an index of the degree of lipemia existing in pathological conditions.

If, however, as our figures as well as those of Gonalous (16) and Shiskin (15) seem to indicate, the total blood cholesterol level in women is such a variable thing, and if the variations in cholesterol are unaccompanied by similar variations in lecithin and fatty acid, it would seem that some of these conceptions of the place of cholesterol in fat metabolism need to be revised.

It may well be that the pre- and postmenstrual increases in the level of blood cholesterol involve an alteration in the ratio of free cholesterol and cholesterol esters and that the menstrual drop in total cholesterol may be a drop in one fraction only.

Our original plan for this investigation included separate determinations of cholesterol and cholesterol esters, but we have been so far unable to get the requisite amount of digitonin. We hope, however, to make these determinations at some future time.

If cholesterol esters play a definite part in fat metabolism, and, as Bloor suggests, the proportion of cholesterol esters to total fat is more or less constant, it would seem that our variation is probably one in free cholesterol. This hypothesis is in accordance with the results of our observations on the relationship of the level of the blood cholesterol to the incidence of respiratory infection.

Indeed, next to the menstrual variations the most striking thing which our data would seem to indicate is the ease with which blood cholesterol may be lowered by slight respiratory infections. Denis' (14) studies lead one to believe that a fall in blood cholesterol is to be expected only in acute infections of a grave character. But none of our subjects were so incapacitated by their colds as to be unable to attend classes during our period of observation. Hence it would seem that, in women at least, there is a suggested

correlation between blood cholesterol level and resistance to infection. It is to be noted, however, that the changes observed in blood cholesterol level at the time of the colds were usually less than the menstrual changes, hence within normal limits of variation. The low values seemed, however, to persist for a longer period than the low menstrual values. Moreover, when the colds persisted through a menstrual period there seemed to be a somewhat decreased tendency to high premenstrual values (Subject F., Period III, Subject Wb.) and a tendency to recovery in the postmenstrual period. Whether or not this is to be connected with an increased resistance as a result of increased cholesterol, it is impossible to attempt to state on the basis of the data at present available. This aspect of the problem suggests also the need of further investigation.

The decidedly increased values of the lecithin-cholesterol ratios which we have observed, usually shortly after the onset of menstrual bleeding, are also interesting in view of the hemolytic properties of lecithin and the antihemolytic properties of cholesterol. Most workers appear to regard the coagulability of circulating blood as unchanged during the menstrual period and ascribe the lack of coagulability of menstrual blood to the addition of an antithrombin from the uterine mucosa. The lipid content of the menstrual discharge seems not to have been investigated, but the variations (12) in the lipids of the uterine mucosa are suggestive. If we may accept Geugenbach's (32) demonstration of choline in menstrual blood, together with the observations of Sieburg and Palzsch (33) on the increased choline content of perspiration and serum during menstruation, as evidence that actual breakdown of lecithin occurs in the uterine mucosa during menstruation, these variations in lipid ratio assume additional significance. It should be noted that while the properties which Macht (34) ascribes to his "menotoxin," notably its ability to inhibit coagulation of blood, check very closely with those ascribed by the investigators quoted above to choline, nevertheless the identity of the two substances would not appear to have been established. Moreover, if we are to regard choline as the menotoxin it is difficult to conceive how a choline-cholesterol complex could be formed, and hence how cholesterol could serve chemically to neutralize the toxicity of the choline.

Again, Kürten (35) regards the suspension stability of red blood cells as dependent on the lecithin-cholesterol ratio. He finds that cholesterol, which lowers the surface charge, brings about an electrical isolation and increases the rate of sedimentation. Lecithin, which is 5 times as active, produces the opposite effect. Hence, he regards increased blood cholesterol with the resultant lowering of surface charges as responsible for an increased resistance to the movement of ions, which leads to retention of salt and water in body tissues. Obviously, we need more data before we can draw absolute conclusions from these observations. Nevertheless, the coincidence of the time of the premenstrual rise in blood cholesterol and the weight increases which often precede menstruation in women, as well as the sharp drops in weight sometimes observed at intervals after the onset of bleeding which may very well coincide with the time of the fall in blood cholesterol level, are suggestive. It would seem that whatever the cause, the retention or discharge of water by the tissues is a more reasonable explanation of such sudden gains or losses in weight than any retention or excretion of solid matter which may take place.

#### SUMMARY AND CONCLUSIONS.

Data from determinations, by the Bloor method, of the lipid content of over 200 before breakfast blood samples, covering twenty-six monthly cycles in sixteen women, are reported. Two of these subjects were on weighed and constant diets, the others ate ordinary college boarding-house fare. It would seem that, while it may be possible to influence general blood lipid levels by feeding, the day to day variations in fasting level are no greater on the uncontrolled than on the constant diets.

The most striking and consistent cyclic alteration in blood lipid content observed was the fall in blood cholesterol, which took place almost invariably during or within a few days of the menstrual period. This was usually preceded or followed by blood cholesterol levels higher than the averages for the individuals concerned. If the average of the observed values for each individual is taken as 100 per cent and each observed value computed in terms of this average, the high points of the curve come at the 124 per cent mark, while the average of the low values observed is 70 per cent, a variation amounting to 54 per cent of the so called normal



value for each individual. Extreme high and low values observed were 157 and 46 per cent respectively of the normals involved. Since the initial rise in cholesterol level, the menstrual fall, and the postmenstrual rise with the secondary fall to approximately the average level of 10 require 2 weeks or more for completion, it will be seen that the blood cholesterol level in women is to be considered as a variable rather than a constant.

The menstrual rise and fall of the blood cholesterol is not consistently paralleled by a similar rise and fall in blood fatty acid and lecithin. In certain cases a tendency to a menstrual alteration in the level of fatty acid was observed. The times at which the lowest fatty acid values were observed usually came later than the low points in the cholesterol curves, sometimes actually on the same days that the highest postmenstrual values for cholesterol were obtained. Hence the fatty acid cholesterol ratios were usually considerably increased (sometimes even doubled) at the time of the menstrual fall in blood cholesterol. The possible bearing of this observation on the theory that cholesterol plays a definite part in fat metabolism is discussed.

Lecithin values tended to be more nearly constant than those for either fatty acid or cholesterol. This meant an increase often reaching 50 to 100 per cent in the lecithin-cholesterol ratios, usually reaching its maximum during the first 2 days of a menstrual period. A possible relationship between the alteration of this ratio and certain changes in the characteristics of menstrual blood is suggested, in view of the supposedly hemolytic properties of lecithin and the antihemolytic properties of cholesterol.

It does not seem fair on the basis of the evidence at present available to consider that any definite time relationships between the monthly variations in lipid metabolism of the ovary and the level of blood cholesterol have been established. It is, however, suggested that the period of retrogression of the corpus luteum may correspond to that of the drop in blood cholesterol.

Decreased levels of blood cholesterol have been observed intermenstrually, associated with slight acute respiratory infections, *i.e.* colds. Since the extent of the fall in cholesterol level is usually rather less than that associated with the menstrual period, it must be assumed to be a variation within normal limits.

It is difficult to make any satisfactory correlation between the

supposed association of the menstrual drop in blood cholesterol with the neutralization of toxins on the basis of the evidence at present available. The need for further histological and biochemical studies of the menstrual phenomena is apparent.

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# THE INFLUENCE OF URETHANE NARCOSIS UPON THE SPECIFIC DYNAMIC ACTION OF GLYCOCOLL AND GLUCOSE IN RABBITS.\*†

BY M. S. GUTTMACHER (BALTIMORE) AND ROBERT WEISS (PRAGUE).

(From the Institute of Experimental Pathology, German University of Prague, Prague, Czechoslovakia.)

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These investigations were made in order to determine the effect of narcosis upon the so called specific dynamic action of food-stuffs. No attempt will be made to review the literature on the subject of specific dynamic action; only such works will be referred to as have a definite bearing upon the problem under study. So far as we have been able to learn, no direct studies have been made on the relationship of narcosis to specific dynamic action. Chance isolated observations have been found in four sources. Aub (1) refers to unpublished work performed by himself and two associates in which they found the effects of thyroid and adrenalin on metabolism were present under urethane anesthesia, even when the stimulation from caffeine and amino acids had disappeared. Weiss and Klein (2) note the rise in the metabolism of rabbits urethanized with 1 gm. of urethane per kilo after the feeding of 10 gm. of glucose. In a paper in the Pawlow Memorial Volume, by Krogh and Rehberg (3), the authors record a single experiment performed on a rabbit narcotized with 1.9 gm. per kilo of urethane in which 4 gm. of glucose were given intragastrically without any rise in the metabolism. Reiss and Weiss (4) record an experiment in which 10 gm. of sugar were given intravenously to a rabbit narcotized with 2.5 gm. of urethane per kilo in which they observed no increase in metabolism.

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*Animals.*

The rabbits used in these experiments were of various breeds. They ranged in weight from 1 to 3 kilos. All of the animals were fasted for 24 hours preceding the experiment. We, of course, realized that because of the structure of the alimentary tract in the rabbit, a true fasting state was not obtainable. So far as possible, all of the work was done on mature animals.

*Narcosis.*

In all of our experiments, urethane "Hoechst" dissolved in 20 to 40 cc. of warm distilled water was given subcutaneously. In the experiments performed in deep narcosis, an interval of 4 hours was allowed to elapse before beginning our observations, so that the body temperature of the animal could become stabilized. To obtain deep narcosis, 3 to 3.2 gm. of the drug per kilo of body weight were employed. Suitable experimental conditions could be established under light narcosis with 1.75 gm. of urethane per kilo. With 1 gm. and 1.5 gm. the animals generally displayed spontaneous movements. In the experiments under light narcosis, we waited 2 to 4 hours after the administration of the narcotic before beginning to measure the metabolism. A careful scrutiny was kept over the animals under light narcosis during the experimental runs, so that movements of even a very slight order might be detected. It was decided not to continue any of the light narcosis experiments longer than 9.5 hours after the injection of urethane, as we had evidence in one case that the anesthesia began to wear off after this time. The drug did not always give identical reactions in all animals. We feel that more variations resulted from differences in the strength of the various lots of the drug than from the degrees of individual variation among the animals. Those boxes of urethane made up chiefly of large translucent crystals seemed not so potent as those containing fine white powder. In one animal, narcotized with the former, we do not feel that even with 3 gm. per kilo we got a truly complete narcosis.

Frequent and careful observations were made to ascertain the condition of nervous reflexes throughout the experiments. But for the one exception just noted, all of the animals receiving 3 gm.

of urethane per kilo were apparently in a condition of areflexia. By areflexia we mean a state in which the animals showed no visible muscular movements due to the irritation resulting from the passage of the stomach tube, the cutting off of an adequate oxygen supply, corneal irritation, the tickling of the nose, the deep insertion of the rectal thermometer, pinching of the hind feet or fore feet, etc. It is interesting to note, however, that the great reflex slowing of the respiratory rate on the rapid introduction into the stomach of the rabbit of a large quantity of fluid at body temperature was present in the animals under deep narcosis also. Under light narcosis with 1.75 to 2 gm. of urethane per kilo a state of complete areflexia was never obtained. With 1 to 1.5 gm. and usually with 1.75 gm. per kilo all the reflexes listed above could be elicited. With 1.9 gm. the licking-swallowing reflex provoked by the stomach tube, the corneal irritation reflex, and the withdrawal of the fore foot on painful stimulation were generally present. These reflexes were less active than when small doses of the drug were employed. In animals which received 1.9 gm. per kilo the hind foot reflex could be obtained only with very strong stimuli. Under light narcosis, discomfort of any type, *e.g.* temporary asphyxia by pinching off the tracheal cannula, may cause movements. At times, these movements consisted merely of winking the eye, at other times of violent contorsive movements of the whole body.

Under light narcosis, the rectal temperature ranged from 36.5–40°C.; under deep narcosis, from 35–39°C. The average temperature under light narcosis was 38°C. and under deep narcosis 36°C. In the deep narcosis experiments, a slightly more rapid respiratory rate was present. These animals had about ten more respirations per minute than those under light narcosis. The deep narcosis, in several instances, persisted 24 hours after the administration of the drug. With some lots of urethane many of the animals died during the night following the experiments. Some of the animals survived more than 5 days. In one case under light narcosis, the effect began to show signs of wearing off after 9.5 hours. Practically all of the animals receiving less than 2 gm. of urethane per kilo of body weight survived more than 24 hours. At the end of this period, they were able to stagger about. In some animals, the degree of narcosis

appeared as complete 24 hours after administration as it did 2 hours after giving the drug.

*Technique.*

The animals were all tracheotomized shortly after the injection of urethane and the largest glass cannula that fitted was firmly tied into the trachea. The animals were then placed in a thermostat which maintained a temperature of between 27° and 30°C. Here they were kept until the experiment was completed. In all the experiments, the Krogh spirometer and the Lovén respiratory valve were used. The oxygen, carbon dioxide, and water vapor displaced from the collecting tubes of soda-lime were measured. The weighing of the soda-lime and calcium chloride glass tubes was performed upon a chemical balance weighing accurately to 1 mg.

A period of 4 hours was permitted to elapse between the injection of urethane for deep narcosis and the measuring of the metabolism. In the light narcosis experiments, this period was from 2 to 4 hours. At this point, we should like to call attention to work done last year by Hawkins and Murphy (5), in which it was found that ethyl urethane had a profound effect upon the carbon dioxide content of the blood. They report, however, that the greatest change occurs within the 1st hour. The increased content persists at a nearly constant level, the average variation between the 3rd and 24th hour being no greater than in normal animals.

In order to establish a standard, two normal periods varying not more than 5 per cent in the carbon dioxide excretion were established before proceeding with our experiments. Experimental periods were accurately measured with a stop-watch. The usual period lasted 12 minutes. The temperatures of the water under the spirometer, of the animals, and of the thermostat were measured during each period. The respiratory rate was counted. At the conclusion of each, or at least of each alternate period, the nervous reflexes of the animal were tested. In the experiments performed under light narcosis, the animals were observed for muscular movements throughout the 12 minute runs. All fluids were introduced at approximately body temperature.

*Observations.**Standard (Basal) Metabolism.*

In our experiments, we found a very meager relationship between the weight of the animals and the metabolism, as was previously noted by Krogh and Rehberg (3). We realize that we were dealing with animals of various breeds and ages. In the carbon dioxide output, we found a variation of 25 per cent per kilo of rabbit, and in oxygen absorption a variation of 35 per cent per kilo. We were unable to note any sex influence on the metabolism of the rabbits. Animals under deep narcosis on which experiments were performed on successive days frequently showed a marked variation in metabolic rate.

*Deep Narcosis.*

Five normal control experiments on animals receiving 3 gm. of urethane per kilo of body weight were performed at various times (Fig. 1). Generally the animals were not disturbed. To one, water was given through a stomach tube; to another subcutaneously, without affecting the respiratory exchange in any way. In one of these five control experiments, the metabolic level remained relatively constant. In the others there was a gradual rise in the oxygen consumption, amounting in two to about 8 per cent. In none was there any actual decrease. Aub, Bright, and Uridil (6) noted in anesthetized cats a rise of 15 per cent above the metabolism of the same animals in an unanesthetized state.

*Light Narcosis.*

Ten control experiments under light narcosis were performed. In the two performed on animals narcotized with less than 1.6 gm. of urethane per kilo of body weight, spontaneous movements were observed. Those receiving 1.75 to 1.9 gm. of urethane per kilo showed a remarkably constant metabolism for at least the first 9 hours. In fact, the metabolic rate showed fewer and less marked variations than in the animals under deep narcosis. The gradual rise referred to above also occurred in this group, but less frequently (Fig. 2). The fact that in one experiment a rise in the metabolism occurred 9.5 hours after narcosis and was



associated with, or at least immediately succeeded by, spontaneous movements of the animals, caused us to discard all increases in this group obtained more than 9 hours after narcotization. We have not done enough of these prolonged experiments to observe whether or not there is a rise in the metabolism coincident with the wearing off of the narcosis, independent of and preceding the appearance of movements. We are under the impression, however, that this is not the case. In animals under light narcosis, the intragastric and subcutaneous administration of water was without effect on the respiratory exchange.

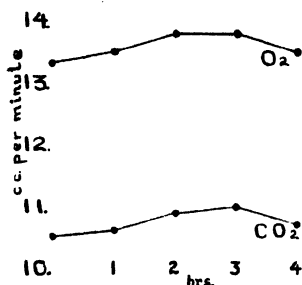


FIG 1.

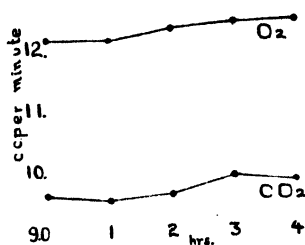


FIG 2.

FIG. 1. Control series. Deep narcosis. Five experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

FIG. 2. Control series. Light narcosis. Seven experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

### *Intravenous Administration of Glycocoll.*

Only three intravenous experiments were performed. In each, 5 gm. of glycocoll (in 30 to 40 cc. of water) were injected into animals under deep narcosis. In none was there a considerable rise in the metabolism. Two other animals were killed in attempting intravenous glycocoll injections. In order to inject anything approaching an isotonic solution of either glucose or glycocoll, such large quantities of fluid were necessary that this project was soon abandoned. The rabbit is so small an animal that appreciable quantities of glycocoll or glucose cannot be administered intravenously without gravely insulting physiological

conditions. The choice lies between the administration of decidedly hypertonic solutions and quantities of an isotonic solution so large as to interfere seriously with the circulatory mechanism.

#### *Subcutaneous Administration of Glycocoll.*

*Deep Narcosis.*—Six subcutaneous injections of 5 gm. of glycocoll were performed under deep narcosis. In one there was a rise of more than 10 per cent in the metabolic rate. This did not begin to appear until 4 hours after giving the glycocoll. In all of the other experiments, there was either a decrease in the oxygen consumption or a minimal increase.

*Light Narcosis.*—Under light narcosis, two injections of glucose and one of glycocoll were made subcutaneously. The two animals receiving 1.7 gm. of urethane per kilo of body weight showed a definite increase in metabolism. The third animal, which showed no increase in the metabolic rate, received 1.9 gm. of urethane per kilo. It appeared unusually sluggish and gave no reflex response. Despite this fact, there was a slight adjustment of the respiratory quotient suggesting, at least, some absorption and utilization.

#### *Intragastric Administration of Glycocoll.*

We felt that of the three paths for the introduction of food constituents, the intravenous, subcutaneous, and gastric, the latter most nearly approached the physiological. We soon found that this method yielded excellent results, and we concentrated all our efforts upon it. This is the part of our work upon which we wish to lay stress; the intravenous and subcutaneous experiments we consider subsidiary.

The introduction of a large quantity of fluid into the stomach of a rabbit by means of a small tube had, in some cases, a very remarkable effect upon the animal. In one animal weighing 1400 gm. death was brought about in this manner. In other animals, the respirations suddenly grew slow and irregular, tending toward periodicity. The temporary discontinuance of the administration generally resulted in a rather prompt return to normal conditions. The animals that showed this slowing of respiration were in both the deep and light narcosis groups. Among the latter,

recovery seemed definitely more prompt. For a short time following this reaction, the reflexes appeared more sluggish.

*Deep Narcosis.*—Six experiments with the intragastric administration of glycocoll and eight with glucose were performed. In all the experiments, there was a definite rise in the respiratory quotient giving evidence of absorption and utilization of the material administered. In four experiments, two glycocoll and two glucose, there was an increase in the metabolism of 10 per cent. In the two glucose experiments, rises occurred after several hours, resembling in all appearances the increases sometimes normally obtained in control experiments under deep narcosis. In one of the two glycocoll-injected animals in which there was apparently a real increase in metabolism, reflexes were unquestionably elicited during the experiment despite the fact that the animal received 3 gm. of urethane per kilo. The increase in the metabolism of the other animal cannot be adequately accounted for. In four experiments, there was a decrease in the consumption of oxygen of more than 10 per cent, a condition that was at no time noted in the deep narcosis control group nor in any of the light narcosis experiments. Of these four, three were glycocoll experiments. The explanation of this lowering of the metabolic rate is not clear to us. The decrease was too great to be accounted for wholly on the basis of isodynamic substitution. In the rest of the experiments, there was observed no conspicuous change in the metabolism. We should like again to reiterate the fact, that in animals in which no visible nervous reflexes could be obtained, we were unable to produce a true specific dynamic increase from either glycocoll or glucose (Figs. 3 and 4).

*Light Narcosis.*—To seven animals under light narcosis we gave glycocoll and to nine glucose, intragastrically. There occurred in none a fall in oxygen consumption. In two experiments, there was an increase of only 9 per cent following the administration of 20 gm. of glucose and 10 gm. of glycocoll respectively. In a third experiment in which glucose was given, the increase was only 8 per cent. In one glycocoll experiment the metabolism remained constant, entirely unaffected by the introduction of this amino acid. In this experiment, no increase of the respiratory quotient occurred; consequently we infer that the glycocoll was not absorbed from the intestinal tract. In each of the remaining

twelve experiments, an increase of more than 10 per cent in the metabolic rate followed the intragastric introduction of glycocoll and glucose. In some of the glucose experiments

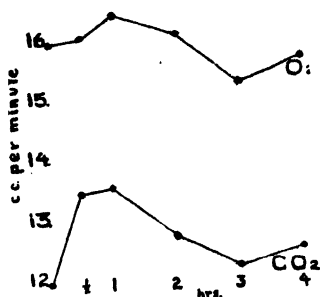


FIG. 3.

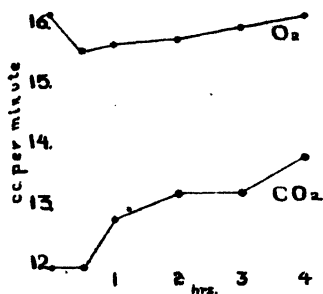


FIG. 4.

FIG. 3. 10 gm. of glycocoll intragastrically. Deep narcosis. Six experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

FIG. 4. 20 gm. of glucose intragastrically. Deep narcosis. Eight experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

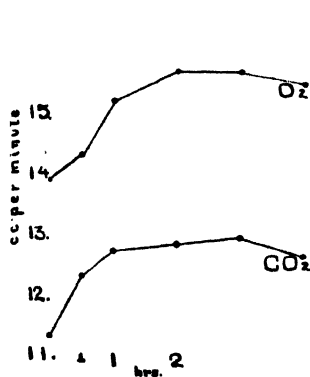


FIG. 5.

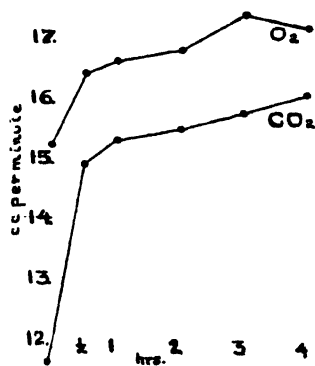


FIG. 6.

FIG. 5. 10 gm. of glycocoll intragastrically. Light narcosis. Six experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

FIG. 6. 20 gm. of glucose intragastrically. Light narcosis. Six experiments. Points plotted are derived by averaging simultaneous readings from one group of experiments.

## PROTOCOL.

	Urethane.	Weight of animal.	Rectal temperature.	Respirations per min.	CO <sub>2</sub> per min.	O <sub>2</sub> per min.	R.Q.	Reflex.	Remarks.
Light narcosis. Control experiment.									
Experiment IV. Animal 38, ♂. Jan. 9, 1926. <i>p.m.</i>	<i>gm. per kg.</i> 1.75 at 9 a.m.	<i>gm.</i> 1000	<i>°C.</i>		<i>cc.</i>	<i>cc.</i>			Very active wink and foot withdrawal reflexes. Pulling out hairs produced head movements instantly. No nose nor mouth response on tickling with feather. 2.40 p.m. 50 cc. warm water by stomach tube; licking reflex followed; no spontaneous movements.
2.09-2.21			37.0	30	7.5	9.6	0.77	+	
2.25-2.37			37.0	30	7.2	9.5	0.76	+	
3.10-3.22			37.0	40	7.5	9.8	0.76	+	
4.10-4.22			37.3	34	7.2	9.7	0.74	+	
5.10-5.22			37.1	32	6.8	9.5	0.72	+	
6.16-6.28			37.1	32	6.9	9.3	0.74	+	
Light narcosis. Glucose.									
Experiment I. Animal 42, ♀. Jan. 15, 1926. <i>p.m.</i>	1.75 at 9.10 a.m.	2400							Reflexes all moderately active. 1.43 p.m. 20 gm. glucose in 60 cc. warm water by stomach tube. Respiration slowed to less than 20 per min. during administration. Licking, writhing reflexes on passage of tube. No spontaneous movements of any kind during experiment.
1.00-1.12			39.0	50	12.7	16.5	0.77	+	
1.15-1.27			39.0	50	12.9	16.5	0.77	+	
2.13-2.25			38.6	33	16.0	18.2	0.88	+	
2.43-2.57			38.8	38	15.7	17.7	0.89	+	
3.47-3.59			39.0	41	15.8	17.5	0.90	+	
4.43-4.55			39.0	58	17.0	18.4	0.92	+	
5.33-5.45			39.2	56	17.3	19.3	0.90	+	
6.33-6.45			39.8	57	16.7	18.7	0.90	+	

## Deep narcosis. Glycocoli.

Experiment VI. Animal 70, ♂. Mar. 19, 1926. p.m.	3.0 at 8.45 a.m.	1950							No reflexes elicited with any stimuli except great slowing of respiration while intro- ducing fluid into stomach. 2.15 p.m. first fraction of 17.5 gm. glycocoli given in 40 cc. water. Respiration slowed to 10 per min.; deep, irregular. Second fraction at 2.50 p.m. in 20 cc. water; respiration only slightly slowed.
1.03-1.15		36.8	42	10.5	14.3	0.74	0		
1.29-1.41		36.8	42	10.7	13.9	0.77			
1.57-2.09		36.7	42	10.5	14.3	0.74	0		
3.15-3.28		36.7	46	11.3	13.3	0.85			
4.15-4.27		36.6	53	11.1	13.1	0.85	0		
5.15-5.27		36.2	50	11.2	12.5	0.89	0		
6.32-6.45		35.9	47	11.6	12.8	0.90	0		

In order to save space the detailed tables of the extensive material contained in this paper are not presented. The full records, however, are on file in this laboratory.

this was much greater, in one 28 per cent. The increase in each case followed almost immediately upon giving the food constituent, so that the form of the resultant curves was different from that of the occasional deep narcosis experiment in which a very much delayed rise occurred. Using the adjustment of the respiratory quotient as an index of the rate of intestinal absorption, it can be seen that the rate is so nearly the same in the deep and light narcosis groups that this cannot possibly be a factor in producing these different types of curves (see Figs. 5 and 6 and Protocol).

*Comparison of Relative Effects of Glycocoll and Glucose.*

The great disproportion in the relative specific dynamic increase in the metabolism after the ingestion of glycocoll and carbohydrate seen in the dog was not found by us to exist in rabbits. Obviously, it is impossible for us to draw exact quantitative deductions as to this relationship. One can infer from the work of Lusk (7) on phlorhizinized dogs that the specific dynamic effect is not dependent upon calorific value. Nevertheless, in order to establish a standard basis for comparison, we gave to some animals approximately equal numbers of calories of glycocoll and glucose. Considering 21 calories as the available value of 10 gm. of glycocoll, we may consider 5 gm. of glucose as its equivalent. No studies of the urinary N, etc., were made to determine whether the glycocoll was totally absorbed. In rabbits, so far as we could determine, the specific dynamic action of these substances is nearly equal. It does not seem surprising that animals living upon entirely dissimilar diets should react to food stimuli in a manner biologically dissimilar. It would be interesting to see whether or not animals could be grouped according to the specific dynamic action of food-stuffs upon their metabolism.

DISCUSSION.

We believe that these experiments show definitely that in the rabbit the specific dynamic effect of foodstuffs is present under light urethane narcosis. When enough urethane is given to produce the condition of areflexia, we believe that it is no longer possible to produce specific dynamic action.

Researches of Tangl (8), showing the presence of the specific dynamic action in curarized dogs, are of great importance. They have tended, however, to divert attention from the nervous system in seeking a solution for the genesis of the specific dynamic action. Wolf and Hele (9) made studies on decerebrate dogs. Only a few experiments were performed, and these were so inconclusive that they throw but little light on the subject.

The Voit-Rubner-Lusk school has maintained the view of a specific stimulation of the individual cells in order to account for this phenomenon. The Zuntz school has conceived of it as the energy cost of digestion. In the light of our investigations, we consider the latter very unlikely. The increased quotient which we obtained in deeply narcotized animals certainly suggests digestion, absorption, and burning; yet we see no specific dynamic increase in the metabolism of these animals.

We believe that our experiments show that the nervous system plays an essential rôle in the problem. We hardly can withstand the temptation of postulating a center somewhere in the medulla to which chemical stimuli are brought and which in turn sends efferent impulses to stimulate individual organ groups to increased metabolism. Grafe (10) refers to experiments made by Freund and Grafe (11) on two dogs in which there was an increase of the metabolism following the administration of sugar after cutting the cervical cord. The experiments do not seem to bear out their conclusions. In one dog (Flora) there was in one experiment (No. 19) an actual decrease; in another (No. 22) the increase amounted to 7 per cent. The experiments on the other dog (Tommy) showed no evidence whatsoever of an increased metabolism as judged by the data of Table I (p. 288) of their paper. New experiments will be required to settle this important point. Other experiments indicating the importance of the nervous system, especially of the autonomous nervous system, are those of Abelin (12), Nakayama (13), Bahn (14), and Gabbe (15). It is not quite safe at this time to attempt to correlate with our findings the results obtained by these investigators. We realize too that although we stress the idea of the importance of the nervous system in bringing about the specific dynamic action, it should be borne in mind that there may be a decreased irritability and reaction of the body cells underlying the decreased



specific dynamic action in deep narcosis. It is only the recognition of the predominating effect of narcotics upon the nervous system that makes us think of this system as being chiefly responsible for the results observed. If this were a toxic action of the narcotic upon the body cells, it is hardly likely that the normal metabolism in the deep narcosis control group would have remained as constant as we have found it.

We find that the very unequal relationship existing between the stimulation from glycocoll and glucose present in the dog does not exist in the rabbit. We suggest the possibility that this relative increase in the potency of carbohydrates in this respect and the diminution in the potency of glycocoll will be found in other herbivorous species.

*Appendix.*—We wish to record five similar experiments made upon dogs, three under urethane and two under amytal narcosis. Because of the scarcity of available animals we were unable to complete a satisfactory series. Apparently 1.5 to 2 gm. of urethane per kilo of body weight together with 6 mg. of pantopon make a rather suitable light narcotic. With 3 gm. of urethane and 6 mg. of pantopon per kilo we had, in one animal, a perfect narcosis. In two other animals, periods of such violent dyspnea occurred that we could not make use of them. In the amytal and urethane deep narcosis experiments which we were able to use, there was an increase of the respiratory quotient following the intragastric administration of glucose without any rise in the metabolic rate. In the dog experiments performed under light narcosis, there was a definite increase in the metabolic rate as well as in the respiratory quotient.

#### CONCLUSIONS.

1. A constant metabolism was observed in rabbits under light urethane narcosis.
2. Rabbits under deep urethane narcosis showed a slightly greater tendency toward a small gradual increase in the metabolic rate.
3. The specific dynamic increase from the ingestion of glucose and of glycocoll is present in rabbits showing nervous reflexes under light narcosis.

4. The specific dynamic increase cannot be produced in animals receiving doses of urethane large enough to do away with all visible nervous reflexes.

5. In the rabbit, under light urethane narcosis, the specific dynamic increase is apparently of equal magnitude after the administration of isocaloric amounts of glycocoll and glucose.

The authors wish to express their gratitude to Professor Biedl for his generous assistance.

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## IRON IN NUTRITION.

### IV. NUTRITIONAL ANEMIA ON WHOLE MILK DIETS AND ITS CORRECTION WITH THE ASH OF CERTAIN PLANT AND ANIMAL TISSUES OR WITH SOLUBLE IRON SALTS.\*

By E. B. HART, C. A. ELVEHJEM, J. WADDELL, AND R. C. HERRIN.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

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In an earlier publication (1) we pointed out that the nutritional anemia induced in rabbits by a whole milk (cow) diet could not be corrected by the addition of inorganic iron ( $\text{Fe}_2\text{O}_3$ ) but that such an anemia could be corrected when in addition to the  $\text{Fe}_2\text{O}_3$  there was added to the diet either fresh cabbage or a cold 95 per cent alcoholic extract of desiccated cabbage or an alcoholic extract of cornmeal. These extracts were practically free from iron. A chlorophyll preparation free from iron fed in the presence of added ferric oxide was also an effective supplement to a whole milk diet as a prophylactic for anemia.

From these experiments we deduced the conclusion that some *organic nucleus* necessary for hemoglobin building was absent or deficient in whole milk. This deduction was in harmony with the generally accepted theory (but for which there was no experimental proof) that there may be a deficiency in whole milk of some nucleus constructed from a multiple of pyrrole groups and which is necessary for hemoglobin building. It was this idea which led us to add a preparation of chlorophyll to whole milk supplemented with  $\text{Fe}_2\text{O}_3$  and which gave us positive results in respect to anemia prevention.

As our work on this problem progressed, we had accumulated data showing that not only were the corn grain and fresh cabbage

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effective agents in the prevention of anemia in the rabbit confined to a whole milk (cow)  $\text{Fe}_2\text{O}_3$  diet, but that lettuce was also especially effective as a preventive of anemia. We had included lettuce in our category of supplements because Evans (2) and associates had used it with marked success as a source of vitamin E and we were not sure at that time but that there might be some direct relation between vitamin E and our problem of milk deficiency and nutritional anemia.

TABLE I.  
*Potency of Fresh Lettuce in the Cure of Nutritional Anemia.*

Rabbit No.	Date.	Diet.	Hemo-globin.	Erythrocytes.	Weight.
	1925		gm. per 100 cc. blood	per c.mm.	gm.
94	June 12	Whole milk only.			365
	" 25		8.9	4,500,000	640
	July 1	Added $\text{Fe}_2\text{O}_3$ (0.015 gm. daily) + 50 gm. fresh lettuce daily.	5.9	3,550,000	780
	" 2				
	" 10		8.5	6,300,000	990
	" 16		9.8	6,275,000	1170
	" 17	Discontinued; in perfect condition.			

We tried to feed dried lettuce in order to demonstrate that drying would not injure its potency, but the dried material seemed to be very unpalatable and the rabbits would not eat the preparation.

In Table I is shown a typical record of the cure of an anemia induced in the rabbit by a whole milk- $\text{Fe}_2\text{O}_3$  diet, by the use of fresh lettuce. The amount of hemoglobin and the erythrocyte count increased almost twofold in a period of 2 weeks.

Before making further attempts to separate the active principle of these plant materials functioning as cures or preventives of anemia on a whole milk- $\text{Fe}_2\text{O}_3$  diet, it was decided to determine whether the *ash* of these materials would be effective. We had tentatively assumed that the preventive principle was of an *organic* character. Our work up to the present has been mainly with *lettuce ash* although the ash of other materials has also been tried.

So successful have been the cures or preventions of nutritional anemia induced by a whole milk- $\text{Fe}_2\text{O}_3$  diet with the ash of certain materials, that we feel certain that the deficiency of cow's milk in respect to its anemia-producing properties is not an organic principle but something of inorganic character resident in the ash of certain plant and animal tissues. These ashes of plant and animal materials were prepared in an electric muffle operating at a temperature of 650–750°C. and for a period of 20 to 30 minutes and were devoid of carbonaceous materials. On occasions the ashes were prepared in gas-heated furnaces at temperatures of a cherry-red. The use of such temperatures must make it certain that we are dealing in these experiments with inorganic materials and not with organic substances.

#### EXPERIMENTAL.

For all the experiments to be described in this paper rabbits have been used. We have also used chicks confined to a special diet as checks on the work with the rabbit, but the ration used and the results secured with chicks will be described in a future publication. The young rabbits were always weaned from the doe at 4 weeks of age and placed on the experimental diet.

The stock ration fed the does during the period of gestation consisted of 100 parts of rolled oats, 10 parts of wheat bran, 1 part of common salt, and whole milk *ad libitum*. Green materials (cabbage, green grasses, or carrots) were given the mother until the young rabbits were about 3 weeks old, but were withheld during the last week of the suckling period. The experimental animals were confined individually in cages fitted with  $\frac{1}{2}$  inch mesh screen bottoms to prevent feces consumption, and were weighed regularly throughout the experiment. They usually weighed 300 to 400 gm. when placed on the experiment. In some cases we have periodically irradiated the experimental animal with ultra-violet light for the purpose of protecting against rickets. We have found that this irradiation had no influence in protecting the animal against an anemia on a whole milk diet. The practice of irradiating our experimental animals has not been general because on the diet used, there has been no direct evidence that our animals suffered from rickets. Sodium citrate, used in our earlier experiments to

prevent the formation in the stomach of large curds and possible gastritis, has not been used in our later experiments. We have found it unnecessary. Our experimental colony has suffered some from intestinal diarrheas terminating in death, but the records presented in this paper are from animals free from such disturbances. Hemoglobin was estimated by the Fleischl-Miescher hemoglobinometer and expressed as gm. per 100 cc. of blood. A discussion setting forth the desirability of expressing the hemoglobin content of blood by a uniform standard will be found in a paper by Elvehjem and Waddell (3). Erythrocytes were determined in an American Standard hemocytometer with Levy counting chamber.

The young rabbits 4 weeks old were given whole milk *ad libitum* until the gm. of hemoglobin per 100 cc. of blood had decreased to 4.7 to 5.8 and the erythrocyte count to 3.5 to 4.5 millions. This generally took a period of from 5 to 6 weeks. At this time the milk was supplemented daily with 0.015 gm. of  $\text{Fe}_2\text{O}_3$  and the lettuce ash. The ash from 5 gm. of ground dried leaf lettuce was triturated with the  $\text{Fe}_2\text{O}_3$  in a small quantity of milk and was fed each morning. This insured a more complete consumption of the supplement. The animals were fed individually. During the remainder of the day the rabbits were allowed an excess of milk.

Table II contains the records of two of the rabbits and is typical of the improvement noted after feeding the lettuce ash to anemic rabbits. Rabbit 180 after showing definite anemic symptoms was fed lettuce ash plus  $\text{Fe}_2\text{O}_3$ <sup>1</sup> directly. Rabbit 164 upon showing similar symptoms was fed  $\text{Fe}_2\text{O}_3$  alone for a period of 19 days to again show that  $\text{Fe}_2\text{O}_3$  fed as such was ineffective in improving the blood stream. Then the lettuce ash was added to the diet. One may readily see from the table that as soon as the ash of lettuce was added there was a steady improvement in the blood stream and after a period of approximately 30 days the amount of hemoglobin and erythrocyte count was normal. After the blood stream became normal, it remained so, provided the ash of lettuce was fed. Rabbit 180 was maintained in perfect condition for 118 days and Rabbit 165 for a period of 111 days. Although discontinued at

<sup>1</sup> The  $\text{Fe}_2\text{O}_3$  used was the same material used in all of our work and was a Baker and Adamson product marked C.P.

TABLE II.  
*Potency of Lettuce Ash in the Cure of Nutritional Anemia.*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
	1925		gm. per 100 cc. blood	per c.mm.	gm.
180	Oct. 5	Whole milk only.			425
	" 16		7.2	4,600,000	540
	" 22		5.9	4,050,000	660
	" 23	Added Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 5 gm. dried lettuce.			
	" 29		6.3	3,950,000	725
	Nov. 6		7.4	4,820,000	770
	" 12		9.3	4,900,000	870
	" 24		9.8	5,150,000	1150
	Dec. 9		10.0	5,970,000	1200
	" 29		12.3	6,650,000	1065
	1926				
	Jan. 6		11.0	6,170,000	1300
	" 13		11.5	6,700,000	1385
	" 20		11.7	6,200,000	1510
	" 29		11.5	6,350,000	1560
	Feb. 10		11.0	6,250,000	1790
	" 18		11.9	6,450,000	1850
164	1925				
	Sept. 29	Whole milk only.			590
	Oct. 9		8.1	5,250,000	660
	" 20		7.0	5,300,000	840
	" 26		7.6	5,200,000	850
	Nov. 5	Added Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily).	7.6	5,100,000	860
	" 8		6.3	3,700,000	860
	" 18				
	" 27		5.1	3,100,000	975
	Dec. 2		5.1	2,770,000	1020
	" 7		5.1	2,920,000	1020
	" 8	Added Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 5 gm. dried lettuce.			
	" 14		7.2	3,520,000	1040
	" 22		7.6	4,000,000	1160
	" 29		7.8	4,200,000	1225



TABLE II—*Concluded.*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
	1926		gm. per 100 cc. blood	per c.mm.	gm.
164	Jan. 6		8.3	4,600,000	1335
	" 13		9.1	5,150,000	1460
	" 20		11.1	6,070,000	1470
	" 29		10.4	5,800,000	1625
	Feb. 10		10.4	5,700,000	1900
	" 18		10.2	5,800,000	2060
	Mar. 1		8.5	5,500,000	2250
	" 17		9.8	5,900,000	2310
	" 29		10.2	6,000,000	2450

that time there is no reason to believe that the normal condition of their blood stream would not have been maintained indefinitely.

Although similar results were obtained with several other rabbits, the rapid improvement of the blood stream and the continued maintenance of their good health produced merely by the addition of the inorganic elements in the lettuce ash with the  $\text{Fe}_2\text{O}_3$  was almost unbelievable. We were slow in being convinced that this was true since our earlier results and those of several other investigators had led to the conclusion that the factor limiting hemoglobin building on a milk diet must be of organic nature. Therefore we immediately started further experiments in order to substantiate our data. More animals were used and greater precautions taken to minimize the rôle of possible genetic factors entering into the experiment.

An entire litter of young rabbits at weaning time was placed directly on the diet plus the various supplements. The preventive type of experiment was used. The litter consisted of eight animals. Each animal was given daily the whole milk plus 0.015 gm. of  $\text{Fe}_2\text{O}_3$ . Two rabbits were given no further addition, two were given the ash of 5 gm. of dried lettuce, two the ash of 5 gm. of dried cabbage, and two the ash of 5 gm. of yellow corn. This gave us an opportunity to compare rabbits from the same litter, all of which had received the same preexperimental feeding. The experiment also made possible a comparison of the potency of the ash of lettuce, cabbage, and corn on an equal basis. Two such

series were started at different times and the animals in each experiment represented a single litter. The results of the entire experiment were exceedingly uniform. The data obtained from the two rabbits in each group and the two groups in each series were so comparable that we present only the data from one rabbit in each group. These results are shown in Table III.

Again it was found that the ash of lettuce was exceedingly beneficial in the prevention of nutritional anemia and the maintenance of a normal blood stream in a rabbit on a whole milk- $\text{Fe}_2\text{O}_3$  diet. Rabbit 301 on the basal diet survived only 148 days, during which period the average amount of hemoglobin was 8.0

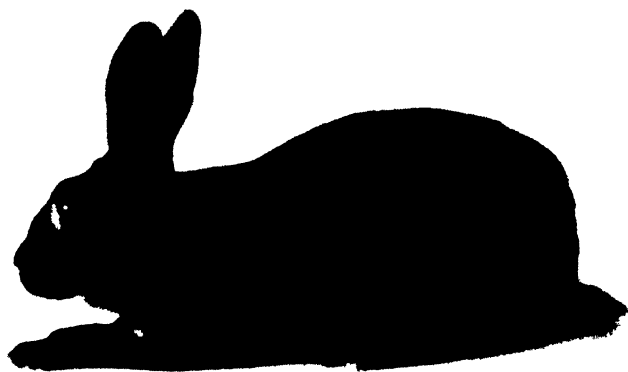


FIG. 1. Rabbit 302, reared on a ration of whole milk- $\text{Fe}_2\text{O}_3$  plus the daily administration of the ash of 5 gm. of dried lettuce. Appears to be a perfect specimen. Hemoglobin normal and erythrocytes per c.mm. about 5,700,000. Started on the ration at 450 gm., and after 9 months on this ration weighed 2400 gm.

and the erythrocyte count 3,810,000. Rabbit 302 receiving in addition to its whole milk- $\text{Fe}_2\text{O}_3$  diet the ash of 5 gm. of dried lettuce made good growth and maintained a normal blood stream over 200 days or 7 months and at this writing is continuing its growth. The average gm. of hemoglobin per 100 cc. of blood during the period was 10.4 and the erythrocyte count 5,950,000. The rabbit appears normal in every respect. Fig. 1 is a photograph of this animal taken September 13, 1926. The results seem to us exceedingly remarkable. This animal received no roughage,

TABLE III.

Rabbit No.	Date.	Diet.	Hemoglobin.	Erythrocytes.	Weight.
Typical record on whole milk-Fe <sub>2</sub> O <sub>3</sub> diet.					
	1926		gm. per 100 cc. blood	per c.mm.	gm.
301	Feb. 17	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily).			530
	" 27		8.7	4,700,000	660
	Mar. 10		7.0	3,000,000	980
	" 19		8.7	4,100,000	1090
	" 26		8.5	4,600,000	1190
	Apr. 5		7.6	3,850,000	910
	" 15		7.2	3,700,000	860
	" 23		5.5	2,600,000	950
	May 3		7.2	3,800,000	1100
	" 12		4.8	2,400,000	1200
	" 24		7.6	4,000,000	1360
	June 8		9.3	4,800,000	1540
	" 21		8.5	4,300,000	1750
	July 5		7.4	4,500,000	1820
	" 15		6.8	3,000,000	1750
	July 24	Dead.			
Typical record on whole milk-Fe <sub>2</sub> O <sub>3</sub> -lettuce ash diet.					
302	Feb. 17	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 5 gm. dried lettuce.			470
	" 27		10.0	6,200,000	680
	Mar. 10		9.3	5,400,000	1000
	" 19		8.9	5,600,000	1170
	" 26		10.6	6,200,000	1320
	Apr. 5		10.0	6,400,000	1420
	" 15		10.4	5,800,000	1460
	" 23		10.8	6,500,000	1560
	May 3		12.1	7,200,000	1650
	" 12		12.1	7,400,000	1670
	" 24		10.2	5,700,000	1730
	June 8		10.0	5,600,000	1800
	" 21		10.4	5,700,000	2020
	July 5		10.4	5,400,000	2200
	" 15		10.2	5,900,000	2310
	" 30		8.9	5,500,000	2280
	Aug. 17		10.0	5,700,000	2450
	" 30		10.2	5,600,000	2510
	Sept. 14		10.4	5,400,000	2520
	Oct. 4		9.3	5,500,000	2520
	" 19		9.8	5,500,000	2400
	Nov. 3		10.2	5,700,000	2390

TABLE III—*Concluded.*

Rabbit No.	Date.	Diet.	Hemo-globin.	Erythrocytes.	Weight.
Typical record on whole milk-Fe <sub>2</sub> O <sub>3</sub> -cabbage ash diet.					
	1928		gm. per 100 cc. blood	per c.mm.	gm.
305	Feb. 17	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 5 gm. dried cabbage.			470
	" 27		8.3	4,400,000	640
	Mar. 10		8.9	4,700,000	840
	" 19		9.6	5,800,000	990
	" 26		8.3	5,000,000	1170
	Apr. 5		10.2	5,700,000	1300
	" 15		10.6	6,000,000	1460
	" 23		10.6	6,200,000	1770
	May 3		9.1	5,800,000	1900
	" 12		9.6	5,600,000	1900
	" 24		11.1	5,800,000	1880
	June 8		10.0	5,600,000	1980
	" 21		9.3	5,400,000	2070
	July 5		9.3	5,400,000	2170
	" 15		9.8	5,800,000	2370
	" 30		10.0	6,000,000	2400
	Aug. 17		9.8	5,500,000	2370
	" 30		8.5	4,500,000	2430
	Sept. 14		10.0	5,600,000	2440
Typical record on whole milk-Fe <sub>2</sub> O <sub>3</sub> -corn grain ash diet.					
295	Jan. 22	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 5 gm. corn.			450
	Feb. 3		8.5	5,825,000	570
	" 10		8.9	6,000,000	600
	" 18		8.1	4,700,000	590
	" 27		6.6	4,500,000	640
	Mar. 9		6.8	4,600,000	810
	" 26		6.3	3,200,000	830
	Apr. 3		6.1	3,100,000	830
	" 13		6.6	3,500,000	930
	May 3		7.8	4,200,000	960
	" 18		7.0	3,800,000	1050
	May 26	Dead.			

having been kept on a wire screen, and yet from all outward appearances, such as sleekness of coat, appetite, etc., appeared as a normal specimen. Reproduction has not as yet been studied.

The ash of 5 gm. of cabbage was nearly as effective as that of lettuce. This is shown in the record of Rabbit 305 (Table III). Although the amount of hemoglobin and erythrocyte count was

TABLE IV.  
*Typical Record on Whole Milk-Fe<sub>2</sub>O<sub>3</sub> + Ash of 10 Gm. of Dried Cabbage Diet.*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
	1926		gm. per 100 cc. blood	per c.mm.	gm.
314	Mar. 9	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 10 gm. dried cabbage.			580
	" 20		7.2	4,300,000	660
	" 29		11.1	6,500,000	860
	Apr. 15		10.6	6,000,000	980
	" 23		11.3	7,000,000	1180
	May 3		10.6	6,500,000	1400
	" 12		9.6	5,900,000	1610
	" 24		8.7	4,700,000	1700
	June 8		10.9	6,000,000	1950
	" 21		10.6	6,400,000	2090
	July 5		10.4	6,100,000	2120
	" 15		10.0	5,700,000	2230
	" 30		10.2	6,100,000	2170
	Aug. 17		10.6	5,800,000	2270
	Sept. 14		9.3	5,100,000	2370
	Oct. 4		9.1	5,000,000	2450
	" 19		10.6	5,900,000	2340
	Nov. 3		9.8	5,700,000	2290

slightly lower for this animal than for Rabbit 302, it is still in our colony, continues to grow, and appears to be normal.

Table IV shows the results secured with Rabbit 314 which was one of the individuals in the later series. This rabbit received the ash of 10 instead of 5 gm. of dried cabbage. It was found that on this higher level of feeding the amount of hemoglobin and the

erythrocyte count were higher than in the case of Rabbit 305 which received the ash of 5 gm. of dried cabbage.

The results obtained from Rabbit 295 as well as those from others receiving the ash of 5 gm. of ground yellow corn clearly indicate that this preparation was impotent. Since it has been shown (1) that yellow corn itself or the alcoholic extract of yellow corn is potent, it appears that the active agent of this seed must be destroyed or inactivated during the process of ashing. It is possible that the prolonged heating necessary for complete oxidation of the carbon in the case of the preparation of the corn ash may be detrimental to the active factor successfully supplementing a milk- $\text{Fe}_2\text{O}_3$  diet. Apparently this active factor is preserved through the ashing process only in the case of certain materials.

Along with our work on plant materials we also tested the potency of dried beef liver and spleen marrow. The spleen marrow was a preparation from Wilson Brothers' laboratory of Chicago for use in the prevention of anemia as suggested by Leake (4). We found both the liver and spleen marrow to be potent if fed at a level of 2 gm. per animal per day as supplements to the whole milk- $\text{Fe}_2\text{O}_3$  diet. The alcoholic extract of equivalent quantities of these materials was also found to be active. We were therefore interested in determining whether these materials retained their potency during the process of ashing. Table V gives the results secured by feeding the ash of 2 gm. of spleen marrow. The results indicate that the ash of spleen marrow may delay the onset of anemia to some extent, but is ineffective over a long period of time. Similar results were obtained in the recovery type of experiment where the anemia was developed before the spleen marrow ash was added. The ash of this material did not bring about a complete cure of anemia as did the lettuce ash. It is possible that the same explanation as was given for the relatively low inactivity of corn grain ash may be applicable to the case of spleen marrow. Such materials on first charring have a dense carbonaceous residue requiring a longer time of heating to secure a carbon-free ash than in the case of lettuce or of cabbage.

All the ash preparations so far shown to be effective in the cure or prevention of nutritional anemia with the exception of the alcoholic extract of cabbage have contained iron in greater or lesser amounts. The dried lettuce contained 0.057 per cent of Fe. This

is equivalent to the addition of 0.0028 gm. of Fe per day when the ash of 5 gm. of dried lettuce was fed; the dried cabbage contained 0.0055 per cent of Fe which was equivalent to 0.00027 gm. of Fe when the ash of 5 gm. of dried cabbage was fed. This is about one-tenth the amount contained in the lettuce; yellow corn contained 0.0029 per cent of Fe and the ash of 5 gm. of this material introduced 0.00014 gm. of iron. Desiccated spleen marrow con-

TABLE V.  
*Typical Record on Whole Milk-Fe<sub>2</sub>O<sub>3</sub> + Ash of Desiccated Spleen Marrow Diet.*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythro- cytes.	Weight.
	1926		gm. per 100 cc. blood	per c.mm.	gm
306	Feb. 17	Whole milk + Fe <sub>2</sub> O <sub>3</sub> (0.015 gm. daily) + ash of 2 gm. spleen marrow.			560
	" 27		7.8	4,000,000	700
	Mar. 10		9.3	5,850,000	1000
	" 19		9.3	5,400,000	1070
	" 26		10.6	6,100,000	1230
	Apr. 5		10.6	6,700,000	1530
	" 15		9.3	5,700,000	1480
	" 23		10.4	6,600,000	1680
	May 3		10.1	6,400,000	1750
	" 12		10.2	5,900,000	1650
	" 24		8.1	4,600,000	1700
	June 8		10.2	5,600,000	1710
	" 21		8.9	4,300,000	1790
	June 22	Dead.			

tained 0.2 per cent of iron and feeding 2 gm. of this material daily introduced 0.004 gm. of Fe.<sup>2</sup> Taking these facts into consideration, it could well be argued that the beneficial effects obtained from them might be due to an addition of iron in a more effective form rather than to the addition of some inorganic substance which allowed a utilization of the Fe<sub>2</sub>O<sub>3</sub>. This seems improbable, because the ash of spleen marrow at the level fed introduced more

<sup>2</sup> The methods used for the determination of the Fe were selected according to the phosphorus content of the material (4).

iron than did the ash of dried lettuce, although the latter was much more effective in the prevention or cure of nutritional anemia. In order to ascertain actually whether or not this were true, the ash of the alcoholic extract of lettuce and of cabbage was used.

It was originally shown (1) that the alcoholic extract of cabbage was practically free from iron and that the addition of this preparation to a whole milk- $\text{Fe}_2\text{O}_3$  diet prevented the development of anemia. In the alcoholic extract of 50 gm. of dried cabbage the amount of iron was so small that it could not be directly determined. It is estimated that there was possibly 0.02 mg. in the extract of 50 gm. or 0.002 mg. in a rabbit's daily allowance. Accordingly if the ash of the alcohol extract of cabbage proved to be potent, one must conclude that something of inorganic nature remained in the ash which favored iron assimilation and its utilization in hemoglobin building. The ash of alcoholic extracts of both cabbage and lettuce was prepared and fed at levels equivalent to 5 gm. of the dried materials. The ash of the alcoholic extract of cabbage was *practically free* from iron. The ash of the alcoholic extract of lettuce contained a *very small amount* of iron; so small that less than 0.1 mg. of Fe was added to the daily diet of each rabbit. Table VI shows typical results obtained by feeding these preparations. In order to include both types of experiment the record of Rabbit 325 receiving the ash of the alcoholic extract of lettuce was taken from the group of animals on the restoration type of experiment, and the record of Rabbit 371 receiving the ash of the alcoholic extract of cabbage from the preventive type of experiment. The record of Rabbit 325 shows the characteristic improvement in the blood stream when a potent preparation is added. The record of Rabbit 371 shows the continued maintenance of a normal condition of the blood stream when fed an active preparation. Therefore it seems logical to conclude that these inorganic preparations do not necessarily add iron in a form more easily assimilated or are potent because they increase the iron content of the ration, but that they add some factor which favors the utilization of the  $\text{Fe}_2\text{O}_3$ .

The results of these experiments seemed quite conclusive to us but we felt that a study of iron salts other than  $\text{Fe}_2\text{O}_3$  would further strengthen our conclusion. Very recently Mitchell and Schmidt (5) have used soluble iron salts (apparently as purchased) in the



TABLE VI.

Rabbit No.      Date.      Diet.      Hemo-  
globin.      Erythro-  
cytes.      Weight.

Typical record of cure of nutritional anemia with whole milk- $\text{Fe}_2\text{O}_3$  +  
ash of alcoholic extract of lettuce diet.

	1926		gm. per 100 cc. blood	per c.mm.	gm.
325	Mar. 27	Whole milk only.			300
	Apr. 5		5.5	4,300,000	380
	" 6	Added $\text{Fe}_2\text{O}_3$ (0.015 gm. daily).			
	" 13		5.1	3,400,000	410
	" 15	Added ash of the alco- holic extract of 5 gm. lettuce.			
	" 21		7.4	5,000,000	500
	" 28		11.9	7,000,000	530
	May 7		10.4	6,800,000	550
	" 14		8.7	5,000,000	620
	" 22		11.9	6,300,000	730
	June 7		10.2	5,600,000	800

Typical record of prevention of nutritional anemia with whole milk-  
 $\text{Fe}_2\text{O}_3$  + ash of alcoholic extract of cabbage diet.

371	May 17	Whole milk + $\text{Fe}_2\text{O}_3$ (0.015 gm. daily) + ash of alcoholic ex- tract of 5 gm. dried cabbage.			360
	" 19		8.9	5,700,000	420
	" 26		9.3	5,800,000	590
	June 2		10.2	6,400,000	720
	" 10		10.8	5,800,000	810
	" 23		8.9	5,400,000	940
	July 8		10.4	6,300,000	1210
	" 21		9.3	5,000,000	1420
	Aug. 4		9.3	5,500,000	1270

cure of nutritional anemia in rats induced by milk diets and have come to the conclusion that hemoglobin building is a matter of availability (solubility) of the iron salt. The first iron salt to be used by us was ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), here designated No. I. This salt was from ordinary stock in our storeroom and labelled iron sulfate (ferrous) c.p. It was fed at a level which made

the daily iron intake equal to that present in 0.015 gm. of  $\text{Fe}_2\text{O}_3$ . By analysis it was found that 40 mg. were necessary to equal in iron content 15 mg. of  $\text{Fe}_2\text{O}_3$ . Table VII gives the record of

TABLE VII.

*Typical Record of Apparent Cure of Nutritional Anemia by the Use of Unpurified  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. I).*

Rabbit No.	Date.	Diet.	Hemoglobin.	Erythrocytes.	Weight.
	1926		gm. per 100 cc. blood	per c.mm.	gm.
287	Jan. 6	Whole milk only.			520
	" 25		7.2	4,475,000	830
	Feb. 3		6.6	5,000,000	850
	" 10		7.0	4,600,000	820
	" 15		5.7	4,200,000	920
	" 24		4.2	2,950,000	980
	" 26	Added $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (No. I) (0.050 gm. daily).			
	Mar. 3		7.6	4,150,000	1040
	" 11		9.1	5,000,000	1170
	" 19		10.2	5,925,000	1280
	" 30		11.1	5,250,000	1310
	Apr. 7		11.9	7,100,000	1320
	" 16		11.1	6,500,000	1520
	" 21		10.4	6,200,000	1720
	" 28		10.2	5,800,000	1780
	May 5		10.4	6,200,000	1370
	" 14		9.8	5,600,000	1470
	" 26		10.6	6,000,000	1610
	June 7		10.2	5,700,000	1720
	" 16		10.2	5,800,000	1980
	July 8		9.8	5,500,000	2080
	" 21		9.6	5,400,000	2120
	Aug. 11		9.3	5,400,000	2280
	" 30		8.1	4,500,000	2380
	Sept. 16		8.5	4,600,000	1630
	" 20	Dead.			

Rabbit 287 which was fed  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. I as purchased) after the characteristic anemic symptoms had developed. The results show that the improvement was quite as rapid as that of rabbits receiving the lettuce ash. The improved condition was

maintained for a considerable length of time (4 months) although after that time the blood picture seemed to deteriorate and finally death ensued. Similar results were secured with several other rabbits.

The rapid improvement shown in the rabbits receiving  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. I) was very surprising to us. We had recognized that the  $\text{Fe}_2\text{O}_3$  we used was insoluble in water but had assumed that it would be somewhat soluble in the HCl of the stomach, and therefore believed it made little difference whether  $\text{Fe}_2\text{O}_3$  or the more soluble iron salts were used. The difference in the availability noted between the  $\text{Fe}_2\text{O}_3$  and the  $\text{FeSO}_4$  led us to study the solubility of the  $\text{Fe}_2\text{O}_3$  in various concentrations of HCl. Thus 50 mg. of  $\text{Fe}_2\text{O}_3$  were weighed out and placed in 100 cc. of 0.02 per cent HCl, shaken vigorously, and incubated at  $37^\circ\text{C}$ . for 5 hours. The remaining insoluble  $\text{Fe}_2\text{O}_3$  was then filtered off on a Gooch crucible provided with a pad of ashless filter paper. The filtrate was tested for the presence of iron by the Thomson method (6). No trace of iron could be detected. Similar tests were repeated using 0.2 per cent HCl and 2.0 per cent of HCl. Even these stronger concentrations of HCl did not dissolve the slightest trace of iron from the  $\text{Fe}_2\text{O}_3$ .

The extreme insolubility of  $\text{Fe}_2\text{O}_3$  reverted our attention to the ash of lettuce. Possibly the extreme potency of the ash of lettuce was due to the presence of more soluble iron salts rather than to some separate factor as was suspected. We therefore tested for the solubility of any iron compounds in the lettuce ash in the same way as was done with the  $\text{Fe}_2\text{O}_3$ . Here too it was found that no detectable trace of iron was dissolved from the ash even with 2 per cent HCl, incubating for 5 hours at  $37^\circ\text{C}$ . These facts seem to be further proof that we are not adding a more available iron compound in the ash of lettuce but that some additional factor effecting the utilization of  $\text{Fe}_2\text{O}_3$  becomes operative. It must be remembered, however, that such experiments prove nothing as to what may take place in the digestive tract with such insoluble iron compounds as  $\text{Fe}_2\text{O}_3$  or the iron compounds of lettuce ash.

Before definitely accepting the hypothesis of factors of inorganic character additional to iron salts as operating in hemoglobin building, it would be necessary to show that highly purified soluble salts of iron such as  $\text{FeSO}_4$  would be non-potent or much less

potent than the purchased, but impure  $\text{FeSO}_4$  (No. I). The rabbits showing the rapid improvement in their blood stream from the use of a soluble iron salt (Table VII) were given a purchased  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. I) which we had not attempted to purify in any way. This  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was far from pure and contained a number of impurities by actual test and specification. It was entirely possible that its potency was due to the presence of impurities of unknown character and not due to its greater solubility. Consequently soluble iron salts were prepared using all precautions to eliminate any possible impurities. A large number of such salts has been prepared using many different methods but only two of the methods will be described in this paper.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. II) was prepared by dissolving pure (?) iron wire in pure (?) dilute sulfuric acid. An excess of iron was added and the solution boiled until no further iron would dissolve. The solution was filtered immediately into an Erlenmeyer flask, stoppered with a Bunsen valve, and 95 per cent alcohol added to precipitate the  $\text{FeSO}_4$ . When the precipitation was complete the  $\text{FeSO}_4$  was filtered off and washed several times with 95 per cent alcohol in an atmosphere of  $\text{CO}_2$ . The precipitate was dried and kept in a tightly stoppered bottle.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. IX) was prepared in a similar manner using so called pure iron wire and a pure quality of sulfuric acid. The  $\text{FeSO}_4$  was precipitated and washed in the cold with glacial acetic acid. All the prepared salts were fed daily at such levels that the iron intake would be equivalent to that in 15 mg. of  $\text{Fe}_2\text{O}_3$ . Some of the salts were fed directly in the milk and others were dissolved in a definite amount of distilled water and added to the milk as a solution.

The inferiority of *purified* soluble iron salts when added to the whole milk diet as compared to the ash of lettuce plus  $\text{Fe}_2\text{O}_3$  is readily seen when an inventory is taken of the records of all the animals receiving these salts. A total of twenty-two rabbits has been fed various purified soluble iron salts. Of that number not one has been benefited to an extent comparable to the rabbits receiving the ash of lettuce. These preparations were unable to maintain the rabbit for a long time and the hemoglobin and erythrocyte counts have not been maintained at high levels. The records were, however, somewhat better than those obtained by the addition of

$\text{Fe}_2\text{O}_3$  alone. Table VIII gives a typical record of one of the animals receiving the purified  $\text{FeSO}_4$  (No. II). Table IX gives the record of two of the animals receiving the purified  $\text{FeSO}_4$  (No. IX). Rabbit 339 (Table VIII) on the prevention type of experiment received  $\text{FeSO}_4$  (No. II) from the beginning of the experiment. Rabbit 409 on the recovery type of experiment received  $\text{FeSO}_4$  (No. IX) after showing distinct anemic symptoms. Rabbit 412

TABLE VIII.

*Record of Results Secured by Use of Whole Milk + a Purified  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. II).*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythro- cytes.	Weight.
	1928		gm. per 100 cc. blood	per c.m.m.	gm.
339	Mar. 31	Whole milk only. Added 0.050 gm. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (No. II) daily.			370
	Apr. 5				440
	" 13		10.2	6,200,000	520
	" 21		10.4	6,500,000	530
	" 28		10.2	6,400,000	520
	May 5		7.6	4,600,000	630
	" 12		7.6	4,500,000	680
	" 21		7.8	5,100,000	790
	June 7		8.1	4,800,000	930
	" 16		8.1	5,000,000	990
	" 24		8.9	4,500,000	1010
	July 8		8.9	4,800,000	1060
	" 21		7.2	3,400,000	1150
	Aug. 11		6.6	4,000,000	1150
	" 30		6.8	3,400,000	1130
		Dead.			

was fed  $\text{FeSO}_4$  (No. IX) after a prefeeding of  $\text{Fe}_2\text{O}_3$ . These records show that although purified soluble iron salts are superior to  $\text{Fe}_2\text{O}_3$  in the prevention and cure of nutritional anemia, they are not as effective as the ash of lettuce- $\text{Fe}_2\text{O}_3$  addition to milk nor as effective as an impure  $\text{FeSO}_4$  (such as No. I). It may, of course, be possible that soluble iron salts can be purified to even a greater degree than we have accomplished so far. It may then be possible to show that, when these purified salts are fed to rabbits whose store of the hypo-

thetical factor influencing hemoglobin building is more effectively depleted, severe anemia will develop as has been shown to be the case with a  $\text{Fe}_2\text{O}_3$  addition. The problem is being studied further.

TABLE IX.

*Record of Results Secured by Use of Whole Milk + a Purified  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (No. IX).*

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythro- cytes.	Weight.
	1926		gm. per 100 cc. blood	per c.mm.	gm.
409	July 9	Whole milk only.			410
	Aug. 13		5.9	3,100,000	580
	" 14	Added 0.040 gm. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (No. IX) daily.			
	" 25		8.3	5,100,000	630
	Sept. 16		9.3	5,200,000	840
	Oct. 1		9.6	5,000,000	1060
	" 11		8.1	4,400,000	1140
	Nov. 3		8.1	4,700,000	1300
	" 11		8.1	4,500,000	1380
	" 23		8.1	4,100,000	1380
	Dec. 3		7.0	4,300,000	1420
	" 16		6.8	4,000,000	1460
412	July 14	Whole milk only.			510
	Sept. 7		5.3	3,500,000	970
	" 8	Added $\text{Fe}_2\text{O}_3$ (0.015 gm. daily).			
	" 16		5.9	3,000,000	1040
	" 17	Added 0.040 gm. daily of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (No. IX).			
	" 24		7.8	4,200,000	1140
	Oct. 4		7.2	3,800,000	1230
	" 11		8.9	4,500,000	1370
	" 18		9.1	5,000,000	1340
	Nov. 3		6.3	3,200,000	1350
	" 11		6.8	3,100,000	1410
	" 23		4.8	2,900,000	1500
	" 29	Dead.			

## DISCUSSION.

The early literature on the utilization of inorganic iron in hemoglobin building is a mass of contradiction and in confusion. The recent contribution to the subject by Mitchell and Schmidt (5), who differentiate between soluble and insoluble iron salts as the key to an explanation of this confusion, would be more secure had they given us evidence that the soluble iron salts used were iron salts only and free from impurities. The status of our own work in this direction makes this phase of the subject of the utmost importance.

Differentiation in the effectiveness of iron salts in anemia on the basis of their solubility is not entirely new. In 1897 Hausermann (7) concluded that small amounts of inorganic salts (he used ferric chloride) may be absorbed and perhaps used to some extent for the production of hemoglobin, but is not nearly so efficient as that contained in natural foods. Bunge (8) has commented upon the insoluble character of the iron of egg yolk and spinach. Not all recent investigators in this field have secured positive results in reference to hemoglobin building with soluble iron salts. Recently Williamson and Ets (9) working with rats and using iron citrate as an adjunct to a synthetic diet showed that the iron was absorbed but that it did not increase the hemoglobin building. On the other hand Scott (10) working with rats on a white bread-whole milk diet did secure results indicating an improvement in hemoglobin formation through the use of inorganic iron salts. However, when he substituted palm kernel oil for the natural fat of the milk he did not obtain such successful results and concluded that the milk fat or something associated with it is necessary for blood regeneration in the presence of either organic or inorganic iron.

The indications at present are that if soluble iron salts are purified their potency in respect to hemoglobin building is reduced. Whether this property can be completely eliminated as we approach complete purity, is a matter for further experimentation. The status of the animal's reserves in respect to hemoglobin-building materials, however complex these factors may be, will also be a matter of the greatest importance in further experimental inquiry.

The fact that the lettuce ash added to a whole milk- $\text{Fe}_2\text{O}_3$  diet is

either a preventive of nutritional anemia or a cure of the deficiency is a difficult fact to circumvent if the matter is a question of solubility of iron salts only. The iron of the ash of lettuce was as insoluble in 2 per cent HCl maintained 5 hours at 37°C. as was that of the  $\text{Fe}_2\text{O}_3$  preparation used, but one should certainly view with caution such data as evidence of what may actually take place in the digestive tract. Incubation at 37°C. for 5 hours of a 2 per cent HCl suspension of  $\text{Fe}_2\text{O}_3$  in the presence of the ash of lettuce did not show an increased solubility of the iron. In fact no iron went into solution. Nevertheless, it should not be concluded from such data that  $\text{Fe}_2\text{O}_3$  such as we used is insoluble in the digestive tract. In all probability  $\text{Fe}_2\text{O}_3$  added to the milk as an extra source of iron actually does function. For example, the daily addition of 50 gm. of fresh cabbage to whole milk will not protect against nutritional anemia unless  $\text{Fe}_2\text{O}_3$  is added (1). The cabbage adds some iron but not nearly so much as an equal weight of lettuce which will protect against nutritional anemia without the  $\text{Fe}_2\text{O}_3$  supplement. Further, the fact that an ash from the alcoholic extract of cabbage practically free from iron was a potent factor in making the system whole milk- $\text{Fe}_2\text{O}_3$  a cure or preventive of anemia indicates unknown inorganic factors or unrecognized forms of iron produced by unknown catalysts as factors in this problem. It does not seem from our data that it is a question of mere solubility of iron salts. We leave the answer to this problem for further investigation.

#### SUMMARY.

1. Nutritional anemia induced by a whole milk- $\text{Fe}_2\text{O}_3$  diet can be corrected by the *ash* of lettuce or by the *ash* of cabbage. While alcoholic extracts of these materials as well as the alcoholic extracts of corn are also potent, the ash of corn grain is not particularly effective. During the ashing process the corn grain ash has become partially inactivated. Spleen marrow, while itself definitely potent, loses this potency in part during the ashing process.

2. It is apparent that the deficiency in milk leading to nutritional anemia is of inorganic character rather than of organic nature.

3. The ash of an alcoholic extract of cabbage is also potent. Such extracts are effective in a system of whole milk- $\text{Fe}_2\text{O}_3$ , and are



practically iron-free. The ash of the alcoholic extract of lettuce is distinctly potent, but is not free from iron.

4. *Impure* soluble  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (although labelled c.p.) was an effective agent in the prevention or cure of nutritional anemia on a whole milk diet. Its effect could be interpreted as due to its solubility as compared to  $\text{Fe}_2\text{O}_3$ . However, *purified* preparations of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  were much less effective as correctives of nutritional anemia as compared to the impure salts, but more effective than  $\text{Fe}_2\text{O}_3$ . The necessity of working with highly purified soluble iron salts is obvious.

5. Further investigation must definitely decide whether we are dealing with extra inorganic factors other than iron and the nature of such factors.

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## THE EFFECT OF HEAT AND OXIDATION ON THE NUTRITIVE VALUE OF A PROTEIN.

BY HARRY GOLDBLATT AND ALAN R. MORITZ.

(From the Department of Pathology, School of Medicine of Western Reserve University, Cleveland.)

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Osborne and Mendel (1) concluded that heat alone does not destroy the fat-soluble growth-promoting factor (vitamin A) contained in butter. This was contrary to the conclusions of Steenbock, Boutwell, and Kent (2) and of Drummond (3) who decided that the vitamin A in butter is thermolabile even in the absence of air. Hopkins (4) confirmed the results of Osborne and Mendel (1) and demonstrated conclusively that the vitamin A in butter is not appreciably thermolabile in the absence of oxygen. He showed that the vitamin A content of butter can be greatly reduced by heating to 120°C. and bubbling air through it for 4 hours, and completely destroyed by heating and aeration for 12 hours. Drummond and Coward (5) confirmed the work of Hopkins. The combined heating and oxidation method of Hopkins (4) has been employed to destroy the fat-soluble vitamins in various substances; vitamin A in casein (Drummond and Coward (6)), vitamin A in cod liver oil (McCollum, Simmonds, Becker, and Shipley (7)), the vitamins A and D<sup>1</sup> in cod liver oil (Goldblatt and Zilva (8)), and the vitamin A in whole diet (Tso (9)).

The temperature at which different investigators have inactivated casein by oxidation has varied from 102–130°C., and the period of inactivation, from 24 to 72 hours. A comparison of the nutritive value of casein inactivated at these extremes of temperature, in order to determine whether, in addition to the destruction of the vitamins, some change is brought about in the protein, has not been made. Chick (10) showed that prolonging the purification of casein at a certain temperature does not alter the biological

<sup>1</sup> Vitamin D refers to the antirachitic organic factor.

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properties of this protein when it is incorporated in a diet deficient in vitamins A and D. Two groups of rats which received diets *deficient* in vitamins A and D and containing casein heated and oxidized at between 120 and 130°C. for 36 to 72 hours respectively, showed no appreciable difference in the duration and rate of increase in weight, calcification of bones, or inorganic phosphorus of their blood. But Chick (10) did not compare caseins heated and oxidized for different lengths of time and incorporated in *normal* diets to determine whether any alteration of the protein had occurred as a result of prolonging the process of purification.

It was the purpose of this investigation to determine whether the inactivation of casein at the extremes of temperature used by previous investigators makes any appreciable difference in its nutritive value and thus introduces a source of error in experiments dealing with the testing of diets for growth-promoting properties.

### EXPERIMENTAL.

Piebald black and white rats bred in this institution from stock rats given to us by Professor H. Steenbock, of the University of Wisconsin, were used in this investigation. The breeders were kept on a diet consisting of milk, bread, mixed whole grain (wheat, corn, and oats), lettuce or cabbage, and a mixture of 1 part of "Parlac" (dried whole milk) to 2 parts of whole wheat flour. Litters obtained from these rats were divided at the age of 30 days into two groups of corresponding sex and weight. Every rat was kept in a separate wire mesh cage 25 × 20 × 15 cm. with removable perforated false bottom. Like Soames (11), we have found that a rat grows better and is less liable to infection when it is alone.

The basal diet given to the experimental animals was constituted as follows:

Technical casein (Merck).....	20 gm.
Wheat starch.....	50 "
Crisco.....	15 "
Vegex (marmite) for vitamin B.....	5 "
Decitrated lemon juice (for vitamin C).....	5 cc.
Salt mixture (McCollum, No. 185 (12)).....	5 gm.
Distilled water.....	50 cc.

The percentage of the casein in this diet is well above that (15 per cent) which was established by Osborne and Mendel (13) as the minimum proportion required for the promotion of normal growth, and is about the proportion of this protein in the normal diets used by most investigators. A comparison of the nutritive value of caseins oxidized at 110 and 130°C. is being made with the proportion of this protein in the diet at the minimum required for normal growth. The results will be published later.

In addition to the basal ration, in order to supply vitamins A and D and thus make the diets complete in every respect, 7 drops (about 150 mg.) of a potent cod liver oil (Harris) were administered daily by mouth to every rat. This quantity has been found by us to be quite adequate to supply the necessary amount of vitamins A and D and promote normal growth if the remaining constituents of the diet are normal in amount and character (Goldblatt and Moritz (14)).

The only modification of the diets given to the two groups of rats was in the character of the casein. In the diet of Group I the casein, in thin layers, on large trays, had been heated at from 105–110°C. for 36 hours (three periods of 12 hours each) in an oven through which a good current of air was constantly blowing. The casein was raked over thoroughly about every hour. In the diet of Group II the casein was treated in the same manner and for the same length of time, but the temperature at which it was heated was between 125 and 130°C.

The rats were weighed at weekly intervals, and the experiment was continued for 13 weeks, at which time all the animals showed the natural slowing of the rate of increase in weight.

Tables I and II compare the weekly gains in the two groups of male rats from corresponding litters and Tables III and IV compare the females. Mean values are given at the bottom of the tables and these show very little difference between the two groups, but the individual weekly and final figures, although the variation, as usual, is very great, also justify the conclusion that there was no appreciable difference between the growth of Group I and that of Group II, whether male or female. Both groups grew quite normally and actually exceeded Donaldson's growth curves for the corresponding sex. The maximum and minimum

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TABLE I.  
*Males. Casein, 110°C.*

Rat No.	Litter No.	Initial weight.	Weekly weights.												
			1	2	3	4	5	6	7	8	9	10	11	12	13
1545	I	46	63	100	130	161	192	235	238	248	260	272	278	285	292
1546	I	48	67	101	129	158	185	212	221	233	242	245	243	254	252
1547	I	48	73	106	129	162	188	230	227	238	250	258	265	273	281
1550	III	44	66	94	120	149	168	182	206	200	217	220	223	222	228
1563	IV	55	92	134	176	221	251	272	270	270	280	292	286	282	282
1564	IV	52	56	88	130	170	203	235	258	280	287	282	292	310	303
1565	IV	55	75	102	137	174	208	238	258	275	277	292	309	293	294
1574	V	45	53	60	93	118	140	157	180	187	192	205	216	217	218
1575	V	44	33	54	76	97	125	157	178	185	207	223	236	232	236
1576	V	40	58	88	118	148	170	185	207	218	233	242	263	263	260
1577	VI	43	63	83	104	125	143	158	170	180	193	193	193	195	198
Mean weights..		47	64	92	122	153	179	206	219	229	240	248	255	257	259
Mean gains....			17	28	30	31	26	27	13	10	11	18	7	2	2

TABLE II.  
*Males. Casein, 130°C.*

Rat No.	Litter No.	Initial weight.	Weekly weights.												
			1	2	3	4	5	6	7	8	9	10	11	12	13
1537	I	48	70	105	135	164	192	211	234	246	253	265	277	285	288
1538	I	47	70	106	132	161	193	213	233	243	255	265	278	283	290
1539	I	48	70	101	128	156	180	200	220	230	243	247	261	267	276
1541	III	44	59	78	93	121	141	154	167	172	178	187	198	202	205
1560	IV	55	85	127	159	192	213	228	242	270	268	270	290	308	308
1561	IV	52	84	116	161	197	228	242	257	273	275	286	292	288	287
1562	IV	55	81	110	145	181	198	206	230	255	265	278	283	293	287
1568	V	45	49	78	109	132	162	190	217	222	231	221	238	237	240
1569	V	44	51	67	90	110	128	148	172	183	198	210	221	224	226
1570	V	40	45	63	80	85	102	117	148	160	176	194	203	210	215
1571	VI	44	54	67	87	92	103	113	128	140	152	155	158	163	169
Mean weights..		46	65	93	120	145	167	184	204	217	227	234	245	251	254
Mean gains....			19	28	27	25	22	17	20	13	10	7	11	6	3

TABLE III.  
*Females. Casein, 110°C.*

Rat No.	Litter No.	Initial weight.	Weekly weights.												
			1	2	3	4	5	6	7	8	9	10	11	12	13
1544	I	48	73	105	131	143	159	165	181	188	195	203	208	208	214
1548	II	54	83	113	135	150	163	172	182	190	193	195	195	198	202
1549	III	44	68	90	109	128	144	154	163	167	168	172	170	165	168
1572	V	41	58	85	106	123	140	148	160	158	167	169	173	175	175
1573	V	41	56	73	88	103	120	138	145	142	146	151	156	165	165
Mean weights..			46	68	93	114	129	145	155	166	169	174	178	180	182
Mean gains....				22	25	21	15	16	10	11	3	5	4	2	2

TABLE IV.  
*Females. Casein, 130°C.*

Rat No.	Litter No.	Initial weight.	Weekly weights.												
			1	2	3	4	5	6	7	8	9	10	11	12	13
1536	I	52	70	89	106	125	140	147	160	167	172	178	180	183	188
1540	II	51	71	83	78	105	136	153	166	167	172	185	196	194	199
1542	III	44	69	88	112	134	147	161	167	173	180	185	192	188	187
1566	V	41	49	73	89	112	125	143	153	157	166	173	178	179	181
1567	V	41	61	85	108	123	137	142	152	153	161	167	171	172	172
Mean weights..			46	64	84	99	120	137	149	160	163	170	178	183	183
Mean gains....				18	20	15	21	17	12	11	3	7	8	5	0

weights and the distribution of the remaining weights were of about the same order in both groups.

## SUMMARY AND CONCLUSIONS.

1. There was no significant difference as regards growth between two groups of rats which received similar complete diets containing 20 per cent casein oxidized for 36 hours at 110 and 130°C. respectively.

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2. The inactivation of casein by oxidation at 110 or 130°C. for 36 hours did not change the nutritive value of this protein as judged by the gain in weight of rats on a diet containing 20 per cent of such caseins. Both groups grew normally.

3. The various temperatures between 110 and 130°C. at which casein has been heated and oxidized for the inactivation of vitamins A and D in casein cannot have been a source of error in the results heretofore published when the proportion of casein in those diets was well above the minimum.

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# DISTRIBUTION OF UNSATURATED FATTY ACIDS IN TISSUES.

## II. VOLUNTARY MUSCLE OF BEEF.

By W. R. BLOOR.

*(From the Department of Biochemistry, the University of Rochester School of  
Medicine and Dentistry, Rochester, New York.)*

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If the hypothesis is accepted that the unsaturated fatty acids in the animal body represent the early stages in the metabolic breakdown of the food fat, a study of their distribution in the tissues—the types of compound in which they are combined and their quantitative relationships—is a logical step toward the understanding of their later changes in the organism. If it should turn out that these acids besides being concerned in the metabolism of fat as a fuel have other functions which their wide distribution in the active tissue would indicate, such a study becomes of great importance. A beginning in this direction has been made by an examination of the fatty acids of the blood plasma of various animals (1) and of beef heart muscle (2). The present communication contains data on the voluntary muscles of beef, it being felt that in the interest of definiteness it was better to confine the attention at first to the tissues of a single animal.

In beef plasma the largest proportion and the greatest degree of unsaturation of the fatty acids as evidenced by the iodine absorption value was found in the cholesterol esters, the phospholipid fraction containing fatty acids of a relatively much lower degree of unsaturation, while, in this respect, the fat came in between. In beef heart muscle cholesterol esters were not found and the unsaturated acids were combined in largest amount and degree of unsaturation in the phospholipids. The fat (glycerides) while slightly more unsaturated than the ordinary stored fat of the animal did not differ greatly from it (all macroscopically visible fat had been removed). Of the phospholipids more of



the unsaturated acids were found in the cephalin fraction than in the lecithin fraction. The unsaturated acids found in heart muscle were a 4-bond acid presumably arachidonic, in amounts generally not exceeding 6 per cent of the unsaturated acids of any of the compounds, and linolic and oleic acids in varying proportions in the different compounds. 3-bond acids were apparently not present.

#### EXPERIMENTAL.

The muscles used in the present investigation were as follows: Jaw, the muscles dissected out from the cheek, mainly those used in mastication; neck, the first cut after the removal of the head; thigh, the first cut of the 'round'; diaphragm, both the circular costal portion and the crura. All samples were obtained fresh from the slaughter house and worked up while still warm. The procedure for the extraction, separation, and examination of the lipids of this series of muscles was essentially the same as that employed for heart muscle (2) and so need not be given in detail here. The procedure gives a practically complete extraction in a minimum of time and with a minimum of exposure to oxidation. The extracted material was divided into (a) acetone-insoluble—of which that portion soluble in absolute alcohol was called lecithin, the insoluble portion, cephalin—and (b) acetone-soluble which consisted of fat (glycerides) and a small amount of unsaponifiable substance. The acetone-soluble material was divided into three fractions. The fatty acids from each of the fractions was separated into solid and liquid (saturated and unsaturated) portions by the lead-salt-alcohol method of Twitchell (3). The results of the analyses are given in Table I, with a summary of the data from heart muscle included for comparison. For economy of space only one complete analysis is given, the others being listed as averages and variations, and the total number of samples analyzed being indicated by the figures in parentheses.

A special study was attempted of the nature of the unsaturated fatty acid mixture by the method outlined by Ellis and Isbell (4), a separation based on the solubilities in various organic solvents of the bromine addition products of the acids. As the work went on it was found that the sum of the fatty acids in the

various fractions rarely exceeded 60 per cent of their initial weight and it became clear that further study of this procedure as applied to the unsaturated acids of tissues would be necessary before dependable results could be obtained. The losses appeared to fall most heavily on the linolic (2-bond) acid fraction and may indicate the presence in these tissues of an unusual linolic acid such as was found in pig liver by Hartley (5). The examination of the ether-insoluble bromides obtained in this procedure showed that only traces of the precipitate were soluble in benzene, indicating that linolenic (3-bond) acid was present in the skeletal muscles only in minute amounts, if at all, which is in agreement with the results on heart muscle and with the findings of Ellis and Isbell (6) that of all the food fatty acids linolenic is the one least frequently stored in the fat depots.

Free and bound cholesterol in samples of each muscle were determined by precipitation with digitonin in aliquot portions (usually one-tenth) of the extract after removal of the phospholipids. Two determinations were made, the first in an aliquot before saponification and the second in the unsaponifiable matter of the second aliquot after saponification. The first determination would give free cholesterol, the second, total cholesterol from which the bound cholesterol could be determined by difference. For the determination of free cholesterol the ether-acetone solution of the aliquot was evaporated to freedom from acetone, then dissolved in sufficient warm alcohol and an excess of digitonin solution (1 per cent in warm alcohol) added. After standing 24 hours the precipitate was transferred to a weighed centrifuge tube, washed twice with alcohol and twice with ether, then dried and weighed. Fraser and Gardner (7) have shown that fat does not interfere with the precipitation, but of course the precipitate must be washed free of adherent fat before weighing. For total cholesterol the aliquot was freed from acetone and to it was added excess of sodium ethylate, after which it was boiled for 8 hours with a return condenser. To the cooled digestion mixture was added in turn 1 volume of petroleum ether (boiling off below 60°C.) and 1 volume of water; the mixture was shaken, the layers allowed to separate, and the petroleum ether drawn off. Extraction with petroleum ether was repeated twice using each time about half the first volume of solvent. The extracts were united, washed

TABLE I.—*Lipids*

Gm. per 1000 gm. of muscle.

Muscle.		Cephalin.							Lecithin.						
		Weight.	Iodine No.	Fatty acids.	Iodine No.	Solid fatty acids.	Liquid fatty acids.	Iodine No.	Weight.	Iodine No.	Fatty acids.	Iodine No.	Solid fatty acids.	Liquid fatty acids.	Iodine No.
				per cent	per cent	per cent					per cent	per cent	per cent		Bromine IV per cent of liquid fatty acids.
Heart (22)*.	Type.	8.00	102	62	137	16	77	180	9	12.80	84	63	107	21	7.8
	Average.	7.28	101	66	124	14	61	170	6.4	9.13	88	65	116	20	5.64
	Low.	5.83	90	58	80	10	46	135	2.8	6.50	82	59	101	8	5.64
	High.	9.33	110	73	150	20	80	217	11.0	12.80	102	71	139	48	10.00
Jaw (10).	Type.	6.39	90	57	134	22	56	181	6.6	7.08	97	71	117	21	
	Average.	4.46	96	58	136	17	57	181	4.7	6.18	93	63	115	15	4.8
	Low.	2.94	86	41	124	9.5	25	156	3.2	3.92	85	42	101	7.5	2.3
	High.	6.39	110	65	149	25	69	195	6.3	7.70	115	79	126	21	6.4
Diaphragm (7).	Type.	2.92	84	61	135	11	57	171		4.34	89	66	118	11	
	Average.	3.27	83	61	116	18	54	155	5.3	4.28	83	61	110	14	3.6
	Low.	2.71	82	54	102	11	35	131	4.3	2.61	79	49	195	7	3.3
	High.	3.91	84	63	135	32	64	171	6.2	5.56	89	68	112	17	3.9
Neck (7).	Type.	2.18	82	62	124	11	60	170	5.0	3.29	80	54	106	10	4.7
	Average.	2.37	84	59	119	13.6	47.5	155	6.3	3.90	79	62	99	19.3	4.3
	Low.	2.18	74	43	107	6.4	22	137	4.3	1.90	74	54	91	10	4.0
	High.	2.58	97	63	125	26	63	170	8.3	5.00	84	67	127	35	5.0
Round (8).	Type.	1.53	87	52	142	16	41	139	7.1	3.18	89	66	125	13	7.3
	Average.	1.57	87	52	132	18	52	134	7.2	2.65	88	64	115	15.0	6.5
	Low.	0.47	82	44	115	16	41	127		2.26	74	56	99	9.3	
	High.	2.27	90	63	151	20	66	141		3.70	106	66	125	24	

\* The figures in parenthesis indicate the total number of samples analyzed.



with water to freedom from alkalinity, then the solvent evaporated. The residue was taken up with alcohol and the digitonin treatment carried out as above. 24.31 per cent of the weight of digitonide was taken as the weight of cholesterol. The results of this series of determinations are included in Table V.

#### DISCUSSION.

*General.*—The arrangement of the muscles (see Tables I and IV) in the order of their average phospholipid content is also found to be the order of their content of unsaponifiable substance but not of their fat (glyceride). The arrangement appears to be also in the order of their activity or of the amount of work being done, the heart muscle being most continuously and strongly active, the muscles of the thigh and neck in the fattening animal least active, while the diaphragm and jaw muscles, after the heart, are most continuously active. Of the two latter the diaphragm being concerned in respiration would appear to be the more continuously active and therefore to rank above the jaw muscle and next the heart; but from the point of view of work done, the masticating muscles probably outrank those of the diaphragm in the fattening and therefore resting animal in which the work of respiration would be relatively light. A correlation, therefore, seems possible between metabolic activity on the one hand and phospholipid and unsaponifiable content on the other. The more work a muscle is called on to do, the greater is its content of phospholipid and of unsaponifiable substance. No such correlation appears possible in the case of the fat (glyceride) which is probably to be expected, since the fat is present only in the capacity of stored material while the phospholipid and unsaponifiable substance are probably built-in constituents of the tissue.

The same order of arrangement holds for the amounts of unsaturated acids in the phospholipid fractions in the muscle groups (Table IV). The unsaponifiable substance varies with the phospholipids from group to group but no exact relation is perceptible although it follows the lecithin more closely than the cephalin. The values for total cholesterol (Table V) indicate that it also varies in the same direction as the total unsaponifiable matter and phospholipids.

*Proportion of Recoverable Fatty Acids.*—Pure lecithin and cephalin should yield about 66 per cent of fatty acids, the percentage varying somewhat with the nature of the combined fatty acids. For fat the yield would be close to 95 per cent. The percentage recovery of fatty acids in the samples examined averaged as is shown in Table II.

In cephalin theoretical recovery was obtained only from the heart. In the other muscles and especially in the round the values indicate the presence of considerable contamination of the cephalin with other substances, which is explainable by the fact that cephalin thus obtained is a mixture of cephalin and its split products (8). In lecithin the percentage recovery averaged sufficiently close to the theoretical to indicate that the lecithin as separated was fairly pure. The percentage recovery of fatty

TABLE II.  
*Percentage Recovery of Fatty Acids.*

	Cephalin.	Lecithin.	Fat I.	Fat II.	Fat III.
Heart.....	66	65	81	85	80
Jaw.....	58	63	85	84	81
Diaphragm.....	61	63	80	94	85
Neck.....	59	62	83	91	76
Round.....	52	64	73	83	86

acids averaged about 82 per cent for all the fat fractions, leaving about 13 per cent unaccounted for, of which the unsaponifiable substance would constitute only a small part. Part of this difference may be accounted for as analytical error but there are undoubtedly other factors to be considered as for example the presence of short chain acids which would result in a lower percentage of recoverable fatty acids. Thus, in their investigation of the stored fat of hogs, Ellis and Isbell (4) found myristic acid in all their samples and most in the fat which was formed by synthesis from carbohydrate. The discrepancy between actual and theoretical fatty acid content in the fat fractions is greatest in the last fraction in which the short chain glycerides would tend to accumulate because of their greater solubility. Other tissue constituents characterized by their solubility in the fat solvents would also tend to accumulate here.

*Constituents of the Fatty Acid Mixture.*

*Solid and Liquid Fatty Acids.*—In the Twitchell lead-salt-alcohol separation of the fatty acids into solid and liquid portions the sum of the solid and liquid acids so obtained was always less than the amount of the fatty acid on which the separation was made. The difference was accounted for in part by the formation of a pitchy residue consisting of lead compounds which are insoluble in boiling alcohol. Part of this substance is soluble in ether and would therefore appear in the liquid acid fractions if the separation were made with ether as in the earlier lead-salt-ether separation. Since the iodine value of the acids so obtained from the pitchy residue has been found to be low (9), these acids would not seem to belong with the liquid (unsaturated) acid fraction and conse-

TABLE III.  
*Solid and Liquid Acids.*

	Cephalin.		Lecithin.		Fat I.		Fat II.		Fat III.	
	Per cent recovery.	Liquid Solid.	Per cent recovery.	Liquid Solid.	Per cent recovery.	Liquid Solid.	Per cent recovery.	Liquid Solid.	Per cent recovery.	Liquid Solid.
Heart.....	75	4.4	73	2.6	63	0.5	67	1.0	81	1.0
Jaw.....	74	3.5	73	4.9	76	1.0	58	1.7	86	1.3
Diaphragm.....	72	3.0	85	5.0	77	1.1	76	1.4	82	1.7
Neck.....	61	3.5	62	2.3	85	0.55	76	1.5	88	2.3
Round.....	70	3.0	66	3.4	73	0.82	81	1.5	81	3.0

quently the lead-salt-alcohol procedure gives a better separation into saturated and unsaturated acids. The sum of solid and liquid acids as separated averaged about 70 per cent of the total acids (see Table III) leaving 30 per cent to be otherwise accounted for. The average data are given in Table III. The percentage of fatty acid recoverable as solid plus liquid is not markedly different in the various fractions except in the last where it is definitely higher. The large amount of the unrecoverable fraction renders its investigation one of the outstanding problems growing from this work.

The ratio of liquid to solid acid is always much higher in the lecithin and cephalin fractions than in the fat. Also as may be

TABLE IV.  
*Distribution of Fatty Acids and Unaponifiable Substance.*

Gm. per 1000 gm. of tissue.

Muscle.	Total fatty acids.										Liquid fatty acids.						Un- saponi- fiable.					
	Cephalin.			Lecithin.			Total phospholipid.				Fat.		Cephalin.		Lecithin.			Total phospholipid.		Fat.		
	Weight.		Iodine No.	Weight.		Iodine No.	Weight.		Iodine No.	Weight.		Iodine No.	Weight.		Iodine No.	Weight.		Iodine No.	Weight.		Iodine No.	
Heart.....	4.36	124	5.93	116	10.30	120	8.45	75	2.66	170	3.14	148	5.81	158	3.30	103	0.882					
Jaw.....	2.58	136	3.89	115	6.47	124	17.21	62	1.47	181	2.26	144	3.73	160	7.39	99	0.690					
Diaphragm..	1.93	116	2.61	110	4.54	113	38.14	66	1.60	150	2.07	130	3.67	166	14.10	96	0.658					
Neck.....	1.40	119	2.42	99	3.82	107	13.04	72	0.67	155	1.04	139	1.71	145	5.97	95	0.459					
Round.....	0.83	132	1.69	115	2.52	120	7.33	69	0.43	134	0.85	136	1.28	136	3.68	96	0.379					



seen from Tables I and IV the liquid acids are much more unsaturated in these fractions.

*Distribution of the Fatty Acids and Unsaponifiable Substance.*—The amount and nature of the different fractions may be seen from Table IV.

*Iodine Values, Oxidation.*—As may be seen from Tables I and IV, the iodine values varied considerably in the different samples of the same muscle and in different muscles. While part of this variation was undoubtedly due to oxidation it is believed that much of it represents natural differences. During the processes of extraction, recovery, and saponification the material was contained in covered vessels and always surrounded by the vapor of the boiling solvent which would exclude most of the air. During the separation of the phosphatides and later of the fatty acids and unsaponifiable substance as well as during the lead-salt-alcohol separation of solid from liquid acid some exposure to air was unavoidable. In the latter case however the material was set in the cold and dark. In the other cases the procedure was made as short as possible and where feasible inert gases, carbon dioxide and nitrogen were used to displace the air. In short, all ordinary precautions to prevent oxidation were taken and operations were speeded up as much as possible. Nevertheless the complete treatment of a sample took ordinarily a week's time, which while less than most of the analyses reported in the literature was still long enough to give opportunity for considerable exposure to air.

As regards the internal evidence of oxidation, considering the type samples, the intact cephalin of heart muscle had an iodine number of 102. Assuming that the iodine absorption was due entirely to the fatty acids (in this case 62 per cent of the molecule) their iodine number should have been  $\frac{100}{62} \times 102 = 164$  as compared with 137—the value found. In jaw muscle the value found was 134 as compared with the theoretical value of 158; in diaphragm the value found was 135 as compared with 138, in neck 124 as compared with 122. This reasoning leads to the same results with lecithin but cannot be applied to the solid and liquid acids because of the unknown effect of the unrecovered pitchy portion. Nevertheless, as noted, some oxidation was

probably unavoidable, and the iodine values reported are to be considered minimal.

The variation in the iodine absorption values of the fractions in different samples of the same muscle and in different muscles may be ascribed in part to the variation in the mixture of solid and liquid acids and in part to variations in the relative proportions of the liquid acids and particularly in the amounts of linolic (2-bond) and oleic acids. Linolenic acid (3-bond) seemed to be lacking while the amount of 4-bond acid averaged about 6 per cent of the liquid acids.

The iodine absorption values of the intact cephalin and lecithin (Table I) in the same muscle were not markedly different from each other although the values for cephalin were generally slightly higher. The iodine values of the fatty acids from cephalin were however always higher than those from lecithin, in which connection it may be significant that the percentage of recoverable acids is somewhat higher for lecithin than for cephalin. The values for the liquid acids of cephalin are also markedly higher than those of lecithin which would point to the possibility that the acids of lecithin were more readily oxidizable than those of cephalin. As to whether the same lecithins and cephalins were present in the different muscles, the iodine values give some, although not very definite, information. Examination of the average iodine values of the intact lecithin and cephalin in Table I and of the total and of the liquid fatty acids in Table IV reveals the same tendency in variation as was noted above for the percentage amounts; *i.e.*, the iodine values tend to be less in the less active muscles. Because of the irregularities this point should perhaps not be stressed, but it is significant that the regular decrease is most marked in the unsaturated acids, being noticeable even in those from the fat. As far as they go the data indicate that not only the amounts of phospholipids but also the degree of unsaturation were greatest in the most active muscles. In this connection it should be mentioned that Costantino (10) found the higher fatty acids from beef heart and beef smooth muscle to have an iodine number of 132 while those from skeletal muscle in the same animal had a value of 116. These values include of course the fatty acids of the glycerides as well as of the phospholipids.

The iodine numbers of the mixed fatty acids of the fat fractions

from the muscle showed that the fat found here was not markedly different from the ordinary stored fat of this animal. Since visible fat had been trimmed away as well as conveniently<sup>1</sup> possible, the results indicate that the fat stored in invisible form was not different from that stored in visible form. The liquid acids of the fat fractions gave iodine absorption values of slightly below 100, indicating that oleic acid was the main unsaturated acid present. Traces of acids giving bromine addition products insoluble in ether were found in most samples of the fat but the amounts were too small for weighing.

TABLE V.  
*Free and Bound Cholesterol.*

Mg. per 1000 gm. of muscle.

	Free.	Bound.	Total.
Jaw 1.....	212	12	224
" 2.....	232	0	232
Diaphragm 1.....	209	0	209
" 2.....	267	33	300
Neck 1.....	59	39	91
" 2.....	84	78	162
" 3.....	360	0	360
Round 1.....	250	45	295
" 2.....	135	0	135
" 3.....	130	20	150
" 4.....	320	0	320

In working with the liquid acids of the fat fractions occasional samples were found which gave iodine values below 90, which may of course be due to incomplete separation of the ordinary solid acids, but since the Twitchell procedure has been found adequate for the separation of the commoner solid acids (palmitic and stearic), it is more likely that the low value is due to the presence of lower fatty acids whose lead salts are appreciably soluble in cold alcohol.

*Free and Bound Cholesterol.*—The results presented in Table V

<sup>1</sup> It should be stated, however, that freeing muscle of *all* visible fat was practically impossible. Of the muscles studied those from the heart and thigh (round) approached most nearly that condition, which may be the reason why the fat fractions in these muscles were the smallest.

show that in these muscles cholesterol esters when present were ordinarily in relatively small proportion and were absent in almost half of the samples. In heart muscle no bound cholesterol was found at all (2), which goes to show that cholesterol esters are probably not essential constituents of muscle. As in heart muscle, occasional samples were met with in which the total cholesterol was less than the bound cholesterol, which is interpreted to mean either that saponification had been incomplete or that some substance precipitable by digitonin had been destroyed by the saponification process.

*Unsapoifiable Substances.*—The weight of unsapoifiable substance in the various muscles (Tables I and IV) varied in the same order as the weights of phospholipids, following however the lecithin more closely than the cephalin. These values are always higher than the cholesterol as determined by the digitonin precipitation (see Table V), agreeing in this respect with the work of other investigators.

*Sphingomyelin.*—Phospholipid insoluble in cold ether was found only in traces in these muscles while in the heart more was found (2). Of the muscles other than the heart the diaphragm appeared to yield most, but the largest amount found even here was 65 mg. per 1000 gm. of muscle.

*Free Fatty Acids.*—As in work on blood and on heart muscle some free fatty acid was always found in the extracts. The amount was rarely higher than 5 per cent of the total fatty acids and the iodine numbers were close to those of the fatty acids from the fat.

In considering the bearing of these results on present beliefs regarding the physiology of the fatty substances attention is directed first to their significance in relation to energy production, the only aspect of fatty acid metabolism which has received much attention. That unsaturated fatty acids were present in muscles has long been known but their nature and relationship there have not been much considered. Leathes' theory of fatty acid metabolism implies that the fatty acids wherever found represent so much fuel, that desaturation is an early stage in their oxidation, and consequently that the unsaturated fatty acids have no other function than to be burned. The phospholipids represent forms of combination of the fatty acids in which they are in

some way more readily oxidizable than when in glyceride combination. Since Leathes was able to show that the liver could desaturate the fatty acids, for this and other reasons he believed that desaturation was a normal function of the liver and that the fatty acids after desaturation were carried by the blood to the tissues to be burned. The form in which they were transported he believed to be that of the phospholipids. The objection raised by the fact that the fatty acids of the phospholipids of blood were of a relatively low degree of unsaturation (1) while these of the cholesterol esters were highly unsaturated might be answered by shifting the synthesis of phospholipids to the tissues. The relationship between metabolic activity and content of phospholipid and unsaturated acid found in the present work would be explained in terms of Leathes' hypothesis on the basis of a factor of safety—having on hand a supply of combustible fuel in proportion to the needs of the tissue. The store would naturally be larger in a tissue which was burning it faster.

The use of the fatty acids for muscular work has been singularly slow of acceptance in the more recent conceptions and theories of muscular contraction, the implication being that only carbohydrate was made use of. Only the obvious fact that in severe muscular work fat must be burned has led in the last year or two to the inclusion of fat as a factor in conceptions of muscular contraction. Nevertheless it has been made evident that carbohydrate is the preferred and under certain conditions possibly the only fuel and it is desirable to consider possible functions of the fatty acids in muscle other than as immediate fuel. Increasing attention is being paid to those elements of the living cell known as mitochondria. Their relation to cell structure and function has been discussed in a recent review by Cowdry (11) who has pointed out that they are universally present in living cells and are especially abundant where there is great protoplasmic activity, as in cytomorphosis, regeneration, compensatory hypertrophy, etc. Directly bearing on the present work is the report of Bullard (12) that the mitochondria (interstitial granules) are more than twice as abundant in heart muscle as in skeletal muscle, thus supporting the chemical findings of Erlandsen (13) that heart muscle has more than twice as much phospholipid as skeletal muscle which in turn is borne out by our results reported

above. Cowdry has noted further that there is a definite relation between mitochondria, cell activity, and intracellular fat. Increased cell activity results in increased mitochondria and decreased fat while decreased oxidation processes result in decreased mitochondria and increased fat. (The step between these normal processes and the pathological process known as fatty degeneration would appear to be easily made. It has long been recognized that the degeneration need not, as at first supposed, mean an actual increase of fatty material at the expense of cell protein but is mainly a rendering visible of material already present in the invisible form. It is only necessary to assume that the normal change of mitochondria to fat as the result of the slowing of cell processes continues with decreasing cell activity until the accumulated fat becomes visible.) As to the chemical nature of the mitochondria all evidence points to their being largely fatty acid compounds and particularly of the unsaturated acids, combined in phospholipid form (lecithin and cephalin). Mayer, Rathery, and Schaeffer (14) have shown a relationship between mitochondria and lecithin content of liver cells and have found that the properties of the mitochondria, their solubility and precipitability, reactions with oxidizing agents and with iodine and bromine indicate a considerable content of unsaturated acids. The observation that they generally stain with osmic acid is further indicative of their content of unsaturated acids. In an earlier paper Mayer and Schaeffer (15) bring evidence to show that in the liver there is a definite relation between cellular activity and phospholipid content. All of which indicates that it is probably necessary to take a wider view of the functions of the fatty substances particularly of the phospholipids and perhaps of the unsaponifiable substances including cholesterol. It appears probable that these substances are constituents of the living protoplasm in somewhat the same sense as protein and that we may apply to them some of the conceptions which have been developed in connection with protein metabolism; *i.e.*, that like cell proteins they may undergo more or less continuous wear and tear and reconstruction, that as fragments of them are broken away in the course of cell activity these portions are replaced and the residues consumed, just as the protein residues are, and furnish energy in the same way; that while some and

perhaps most of the fatty acids of the food are put to immediate use as energy producers another portion is used to replace the wear and tear of the tissue lipid. To what extent the parallelism may be carried is of course unknown. The fatty acids are apparently different from the amino acids in that their synthesis in the animal body is easy while synthesis of the amino acids is very limited in scope. Feeding experiments however indicate that practically the only fatty acids synthesized from carbohydrate are oleic, palmitic, and stearic (4); the more unsaturated acids such as are contained in considerable proportions in the phospholipids are not accumulated in that way. Further desaturation can be accomplished by the liver as shown by Leathes but the extent of the ability of the liver to desaturate is not known and it is possible that the ability of the animal body to synthesize fatty acids may be limited. The conception of the fatty acid compounds as essential constituents of the living tissue allows another interpretation of the variableness of the results given above. The more active the muscle the greater its amounts of essential lipid material—phospholipid and unsaponifiable substances in the form of mitochondria or other structures—and a continually and strongly active muscle such as the heart would have more of these than a periodically but nevertheless strongly active muscle such as the jaw, and these more than practically resting muscles such as those of the thigh. Similarly the variations in the phospholipid content of different samples of the muscle could be referred to differences in the habits of activity of different animals. Amthor and Zink (16) have stated their belief that the more unsaturated acids of the stores of wild as compared with tame animals of the same species may be referred to the greater activity of the wild ones.

#### SUMMARY.

Data are presented on the distribution and nature of the lipids of beef muscle with special reference to their unsaturated fatty acid content. No outstanding qualitative differences could be noted but quantitatively the muscles fall into a definite order as regards their phospholipid and unsaponifiable content which is apparently the order of their activity—the more active the muscle the higher its percentage content of phospholipid and

unsaponifiable substance. No such relation was evident as regards the fat (glycerides) which appears to be ordinary stored fat. The total cholesterol content of these muscles falls into the same order as the phospholipid and unsaponifiable; apparently it also is related to the activity of the muscle. Cholesterol esters are not constant constituents of muscle, being frequently absent and when present being found mainly in small amounts. The bearing of these results on present conceptions of fat metabolism is discussed with especial reference to the cell structures known as mitochondria.

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## CARBOHYDRATE UTILIZATION.

### II. RATE OF DISAPPEARANCE OF VARIOUS CARBOHYDRATES FROM THE BLOOD.\*

By JOHN G. REINHOLD AND WALTER G. KARR.

*(From the Biochemical Laboratories of the Philadelphia General Hospital and the Graduate School of Medicine of the University of Pennsylvania, Philadelphia.)*

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In an earlier paper from this laboratory (Du Vigneaud and Karr, 1925) it was shown that the previous nutritive condition of an animal is important in determining the nature of the blood sugar curve following administration of glucose. The duration of the fasting period preceding the determination of glucose tolerances was particularly important in this direction, and showed decided tendency to lower the glucose tolerance, this effect being increased as the fasting periods were lengthened. Protein or glucose fed before the fast tended to counteract its effects, while glucose given a short time before the glucose test meal greatly increased the tolerance as reflected in blood sugar levels. Similar results when glucose was injected intravenously demonstrated that this effect was not due to a changed rate of absorption from the intestine. It was pointed out in connection with these findings that the reaction of the animal to a period of fasting provides a convenient method of reducing sugar tolerance at will, and in this way becomes a valuable method for the study of glucose metabolism.

In view of the decisive nature of this effect upon glucose tolerance, it seemed important to ascertain the effect of similar treatment upon other sugars, and it was with this primary object, and also the hope that a study of this reaction might throw additional

\* The data presented in this paper were submitted by John G. Reinhold to the faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Master of Science in Physiological Chemistry.

light on the fate of these sugars in the body, that this study was undertaken. The determination of successive sugar tolerances, while essentially the opposing extreme of the fasting treatment, also seemed to offer aid in this direction by furnishing another method for additional comparison of the metabolism of these sugars.

Since most of the pertinent literature has been reviewed in a preceding paper and most of the earlier work was necessarily inaccurate because of the absence of reliable blood sugar methods, discussion will be restricted to the more recent papers dealing with the tolerance (as expressed in blood sugar curves) for sugars other than glucose.

In 1913, Bang and his pupils obtained hyperglycemia from starch in fasting rabbits, but found no increase in blood sugar while digestion was in progress. Shortly after, Hoffman (1914), who was apparently the first to compare the effect on blood sugar of glucose, lactose, galactose, and fructose, observed the last named to be least effective in producing hyperglycemia. Field (1919) obtained curves which showed glucose, maltose, sucrose, mannitol, and lactose to be effective in the order named in raising the blood sugar of normal males. Sucrose and dextrin were found by M. R. Jones (1920) to have only a slight effect on blood sugar.

According to Folin and Berglund (1922) glucose is capable of producing greater hyperglycemia than any other carbohydrate, its superiority in this respect being explained on basis of the relative unsaturation of body tissues for sugars other than glucose, which results in their early removal from the blood.

In the following year, Foster (1923) studied the differences between arterial (finger blood) and venous blood. While glucose produced a higher sugar content in finger blood than in venous, fructose gave a much smaller rise in arterial blood and practically no rise in venous blood, thus showing a greater utilization or storage in the tissues than glucose. Galactose produced high blood sugar values with only slight differences between arterial and venous bloods.

In connection with studies on liver destruction, M. Bodansky (1923) had occasion to determine the tolerance of dogs for various sugars, and found galactose to be most effective in producing hyperglycemia, and fructose least effective, while glucose occupied an intermediate position. He observed a remarkable action of glucose in reducing the hyperglycemia of galactose to a level only slightly greater than that of glucose when given alone, an effect which was absent when fructose was substituted for glucose. Folin and Berglund had previously observed glucose to be effective in abating galactosuria.

20 gm. of galactose were found by Schatti (1923) to have only a slight

effect on blood sugar when fed to normal individuals, causing even less hyperglycemia than lactose or fructose. A somewhat similar result was obtained more recently by Goldblatt (1925) who reported 50 gm. of galactose to produce blood sugar curves similar to those following fructose. From a comparison of these results it appears that galactose is well utilized by man in moderate doses, but that larger doses (around 80 gm.) temporarily exceed the capacity of the human organism to remove the sugar from the blood, with the result that hyperglycemia is produced.

Goldblatt also determined the effect of various sugars on starvation ketosis and found that fructose, sucrose, and maltose as well as glucose showed antiketonic activity, while galactose and lactose did not control ketosis, although they restored the carbohydrate metabolism to its normal condition.

#### *Method.*

Rabbits, averaging a little over 2 kilos in weight, were maintained on a diet of bread, oats, bran, and hay, and in addition, cabbage, beets, or lettuce. The vegetable portion of the diet varied considerably in amount and in variety, depending on the particular availability of the various vegetables at any given time.

In order to eliminate the effect of food intake immediately preceding the experiment, the rabbits were placed in metabolism cages 18 hours before, and deprived of food during this preliminary period which had previously been found not to be long enough to produce any important interfering effects.

To measure the effect of fasting, a 4 day period was used. As pointed out by Du Vigneaud and Karr, this is of sufficient duration to bring out definitely the effect of fasting, yet allows the rabbits to recover readily from the effect of inanition. During the fasting period the rabbits were kept in metabolism cages and allowed free access to water.

While the decreased tolerance observed after fasting may have been due to anhydremia caused by the failure of rabbits to take water, an experiment in which 100 cc. of water were administered daily during a 4 day fasting period, showed an even greater decrease in tolerance, indicating that under the conditions obtaining in this series of experiments, anhydremia was not especially important in the production of lowered tolerance.

The feeding of gelatin before the 18 hour fasting period

was introduced to eliminate the possibility of voluntary fasting on the part of the animal preceding the experimental fast. Since Du Vigneaud and Karr had observed that 5 gm. of gelatin exerted a maximum beneficial effect on glucose tolerance when given 18 hours before the sugar, this interval and dosage were accordingly adopted. It was subsequently observed that gelatin appeared to vary in its effectiveness when used with different sugars, and the study was extended to include these variations. The gelatin used was of the best bacteriological grade, and was given by stomach tube in 20 per cent solution.

The following sugars were used: fructose, Kahlbaum and Difco standardized; galactose, Difco standardized and Eastman; lactose, Baker's C. P.; maltose, Eastman; sucrose, Baker's C. P.; glucose, Merck's (analyzed 90 per cent).

The sugars were given in amounts varying from  $1\frac{1}{2}$  to 6 gm. per kilo of body weight, and were in all cases dissolved in 25 cc. of water and administered by means of a stomach tube, being followed by another 10 cc. portion of water to remove any solution remaining in the tube or funnel. The starch used was Lehn and Fink's best quality potato starch. It was stirred into 25 cc. of water and given by tube, being followed by 10 cc. of water.

Successive tolerances were, whenever possible, compared with corresponding glucose tolerances on the same animal.

Blood for sugar determinations was drawn from the marginal vein of the ear with the aid of xylene, immediately preceding the administration of the carbohydrate to be studied, and subsequently at half hour intervals for  $1\frac{1}{2}$  hours, after which hourly samples were taken. Blood sugar was determined by the Folin-Wu (1920) method.

The results are given in the accompanying charts, in which the curves are in all cases composites of the results obtained from several animals, the number being indicated on the charts.

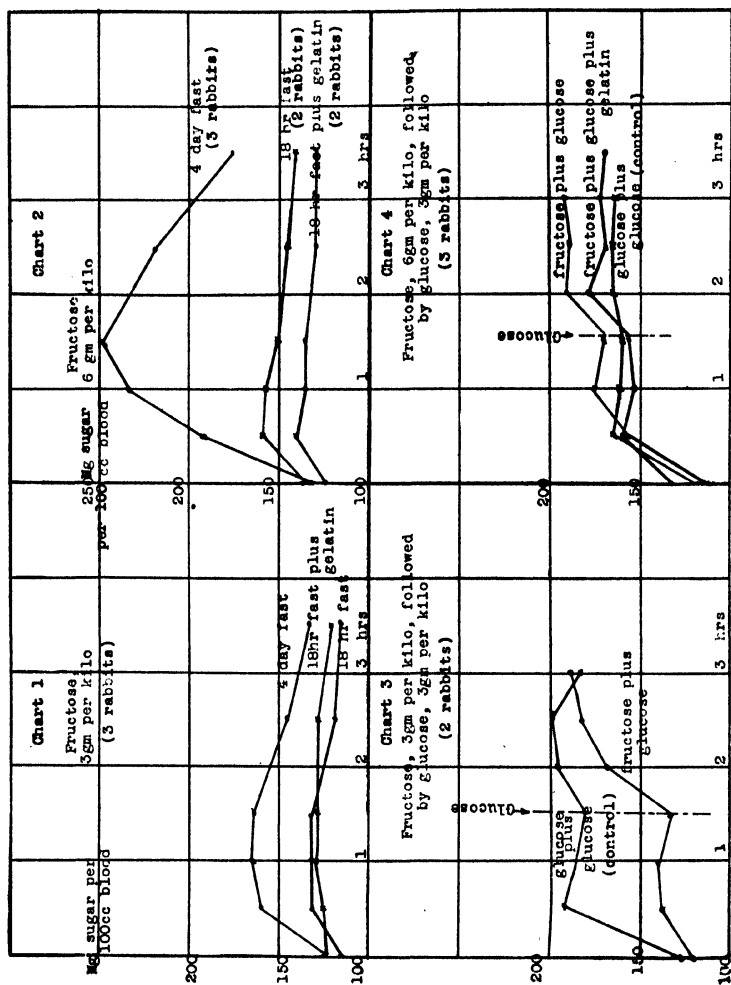
Urine sugars were determined by Sumner's (1925) method, after it had previously been ascertained that the sugars studied would reduce the dinitrosalicylic acid reagent. The urines were compared with a glucose standard.

## EXPERIMENTAL.

*Fructose.*

Fructose (Charts 1 and 2) after an 18 hour fast produced very little rise in blood sugar, and very frequently caused a temporary lowering below the normal level. Fasting decreased the rate of disappearance by 25 per cent in those tolerances in which 3 gm. of fructose per kilo of body weight were given (Chart 1), and 50 per cent in those receiving 6 gm. per kilo (Chart 2). This is definitely opposed to the hypothesis of Folin and Berglund, that the amount of fructose in the blood is controlled through absorption by the tissues. Were this the case, the unsaturation of the tissues for a particular sugar should logically be increased by fasting, with a resultant increase in the rate of disappearance of the sugar from blood, while, actually, as shown by the accompanying charts, the opposite takes place. The alternate suggestion of these authors that fructose is converted into nascent glucose, which is more readily utilized than preformed glucose, seems more likely and is in closer agreement with the results presented in this paper, although Wierzuchowski (1926) has shown that fructose, when injected intravenously at a continuous rate, has an essentially individual and characteristic metabolism, which is not immediately merged with that of glucose.

While there was a definite increase in blood sugar in those experiments in which glucose followed fructose (Charts 3 and 4), the latter sugar tended definitely to reduce the hyperglycemia due to glucose administration. This is best seen in those experiments in which 6 gm. of fructose composed the dose (Chart 4), glucose here causing a rise of only 20 mg. This behavior demonstrates that fructose has the power to stimulate the glucose-removing mechanism, but whether it does this intrinsically as fructose, or because of a similarity of the degradation products resulting from the two sugars, or whether it is the result of isomerization to glucose or its hypothetical active form, cannot be determined from these experiments. The last possibility is least in accord with recent work. It is clear, however, that the two sugars are not equal in their ability to accelerate the disappearance of glucose but that roughly 6 gm. per kilo of fructose produced an effect equivalent to that obtained by 3 gm. per kilo



CHARTS 1 TO 4.

of glucose. In addition these experiments confirm the view that a severe hyperglycemia is not essential to the stimulation of the mechanism involved in the removal of glucose from the blood, although it may be a factor in such an occurrence.

Gelatin fed 18 hours previously shows a slight but definite tendency to decrease the alimentary hyperglycemia resulting from fructose, although it is not as effective as with glucose.

Cori (1925) offers evidence that fructose is absorbed at a much slower constant rate than galactose or glucose. This is supported by the earlier work of Hewitt (1924) and Nagano (1902), although these investigators find a smaller difference between fructose and the other sugars than did Cori. The relatively slight rise in blood sugar values following fructose ingestion can be explained, Cori believes, on the basis of this slowness of absorption. Although this seems the obvious explanation of the behavior of fructose under ordinary conditions, it fails to account for an entirely dissimilar action which results from the administration of 6 gm. per kilo of fructose after a 4 day fasting period. Following such treatment, there is produced a sharp rise in blood sugar to a level well above 225 gm. per 100 cc. of blood, followed by a delayed return to normal. The blood sugar curve in these experiments shows a striking similarity to curves obtained following the ingestion of glucose. It seems difficult to account for this peculiar behavior of fructose, for when the two conditions involved are considered separately, neither seems to have any particularly important effect. At least, these experiments demonstrate clearly that fructose may be absorbed rapidly enough to produce severe hyperglycemia, and that the absence of this phenomenon is not a result of the failure of the sugar to enter the circulation.

Both Hewitt and Cori in assuming absorption to be the determining factor in fixing the blood sugar levels following fructose ingestion have minimized the ease with which fructose is utilized by the organism. Thus Johansson (1909), Lusk (1915), Benedict and Carpenter (1918), and Burger (1921) all found fructose to have a greater effect on heat production following oral administration than did other sugars, with the exception of sucrose. Bornstein and Holm (1922) found for fructose a latent period of only 7 or 8 minutes before oxidation began, in contrast to a delay of 15 to 30 minutes preceding the oxidation of glucose, while



Cori (1926) in his latest paper convincingly shows that fructose is superior even to glucose as a glycogen former. Considering this evidence in addition to that obtained in these experiments, it would seem with fructose that ease of utilization by the tissues would be of greater importance in preventing more than moderate rises in blood sugar under ordinary conditions, although this reasoning is based to a considerable extent on the assumption, as yet unproven, that the fasting effect is not an absorption phenomenon. Apparently, however, the fructose-removing mechanism of the rabbit possesses a quite definite upper limit of maximum activity which enables it to dispose of 3 gm. per kilo of fructose after a 4 day fast with the production of only a mild hyperglycemia, yet whose ability was definitely exceeded by doubling the dose under the same conditions with the resultant increase of blood sugar to a high level.

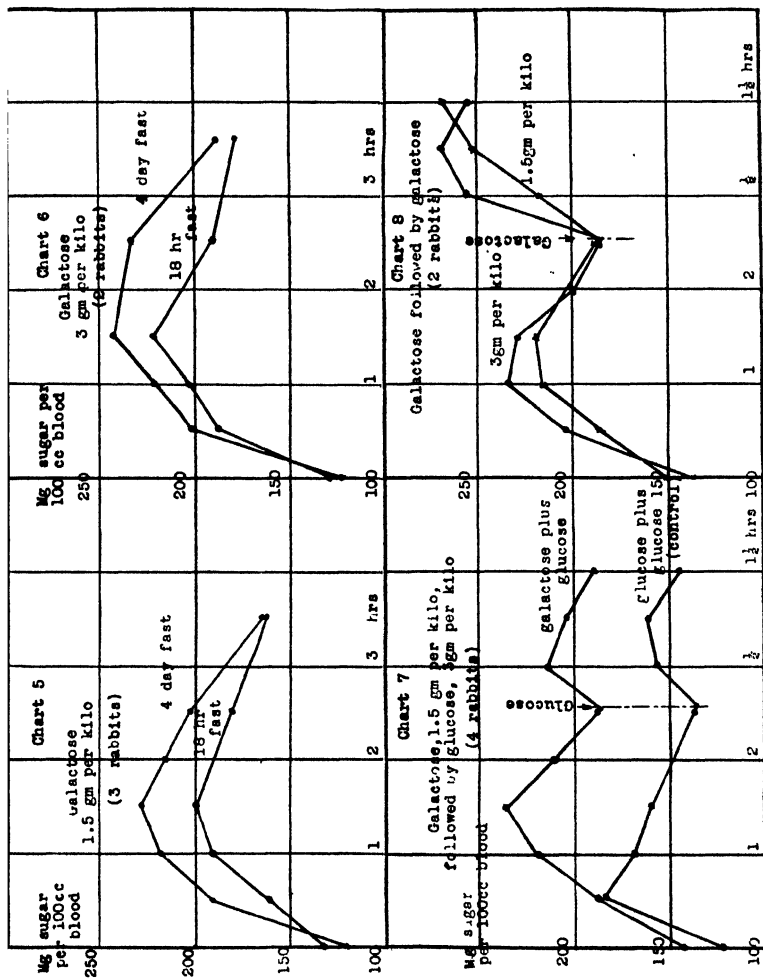
#### *Galactose.*

Galactose caused a greater hyperglycemia than any other sugar studied (Charts 5 and 6) and produced a curve marked by a relatively gradual rise extending over an hour and a half, followed by a very gradual fall. There was still a marked hyperglycemia at the  $3\frac{1}{2}$  hour period.

The effect of fasting on blood sugar curves following administration of galactose was to increase the height of the curve by 20 to 25 mg. per 100 cc., the shape remaining unaltered. Increasing the amount of sugar given from 1.5 gm. per kilo to 5 gm., produced a somewhat greater rise (about 45 to 50 mg. per 100 cc. of blood), indicating that galactose like levulose absorption is also increased by a greater concentration of sugar in the intestine.

Mixtures of glucose and galactose ( $1\frac{1}{2}$  gm. of each per kilo) produced a higher maximum blood sugar than galactose alone (Chart 9), occurring at approximately the same time ( $1\frac{1}{2}$  hour period), but the hyperglycemia produced was of shorter duration, failing to show the lag characteristic of galactose. Blood sugar curves for these mixtures were characterized by very abrupt changes, the initial rise, especially, being very sharp.

Successive tolerances with galactose followed by glucose (Chart 7) showed no increased hyperglycemia following administration



CHARTS 5 TO 8.

of the second sugar, this result corresponding to the effect of similar tolerances in which glucose followed glucose. As pointed out in the discussion of the levulose tolerances, such behavior may indicate a conversion of galactose into glucose or into substances resulting from glucose, or may simply be due to a similarity in structure; that is, possession of a common stimulating group. Galactose, however, shows a greater ability in this direction than does levulose, which could conceivably result from the greater hyperglycemia produced by galactose.

It was interesting to discover whether galactose, when followed by a second dose of the same sugar, would stimulate a mechanism similar to that concerned with glucose in successive doses. Two

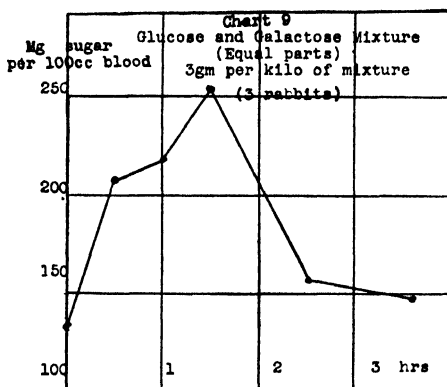


CHART 9.

experiments showed a slight ability in this direction to be present (Chart 8), the rise following the second sugar being perceptibly less than the initial increase.

It has been frequently reported that galactose, following alimentary administration, is as much as 50 per cent excreted in the urine. Determinations made during the course of these experiments showed only a 2 per cent excretion in the urine during the  $3\frac{1}{2}$  hour period of study, thus demonstrating that in the galactose experiments, the kidney is not playing an important rôle in the regulation of blood sugar. The rate of urinary excretion seemed fairly constant for various individuals.

*Lactose.*

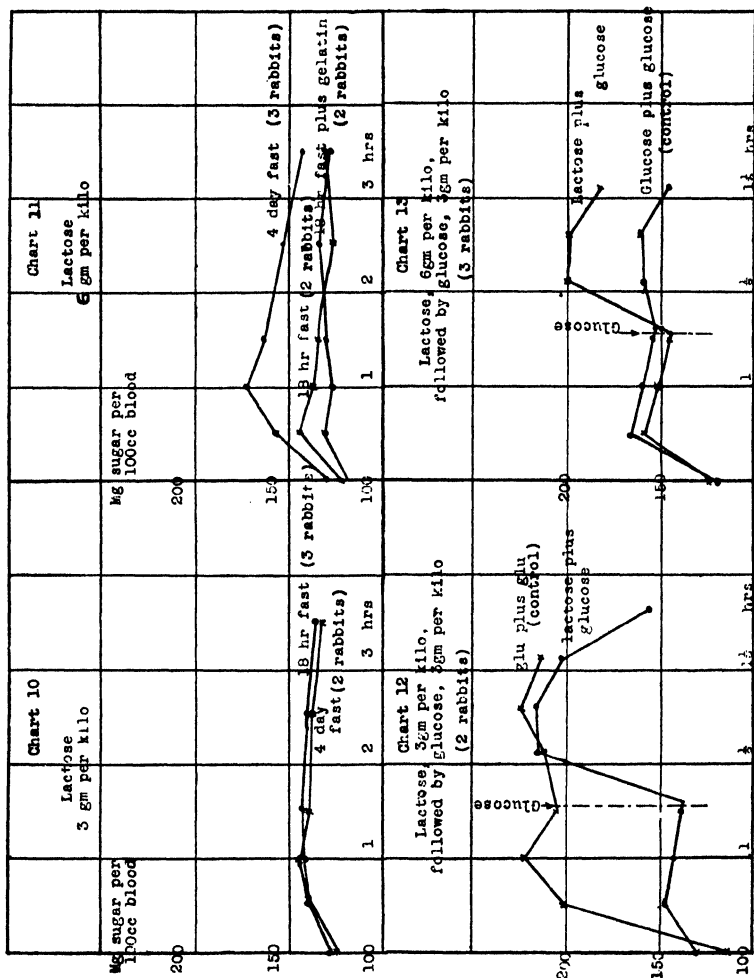
Animals fed lactose showed very small increases in blood sugar level (Charts 10 and 11). Fasting 4 days caused no hyperglycemia in rabbits receiving 3 gm. per kilo, but produced a more marked rise in the 6 gm. per kilo group. While lactose has been assigned a slower rate of absorption than maltose, sucrose, and the monosaccharides, the fact that there is produced an appreciable hyperglycemia under certain conditions (6 gm. per kilo, 4 day fast) would show that slow hydrolysis and absorption is again not the determining factor.

The amount of sugar, as glucose appearing in the urine, following lactose feeding averaged 0.140 gm., which, when compared with the amount of sugar fed to the animal, is negligible, and could have no appreciable effect in reducing hyperglycemia.

Protein given 18 hours previously slightly accelerated the disappearance of the hyperglycemia following lactose administration.

Successive tolerances in which glucose followed lactose (Charts 12 and 13), were characterized by a considerable increase in blood sugar after the second sugar, showing that the glucose-removing mechanism had not been stimulated by the lactose or its hydrolyzed components. This may be due to the failure of lactose to produce important increases in blood sugar level, although as previously pointed out, there is considerable evidence tending to show that hyperglycemia alone is not sufficient to stimulate the glucose-removing mechanism.

The contrast between the behavior of lactose and its component sugars, already referred to under galactose, when given in equivalent amounts under similar conditions, is difficult to explain. Failure of the rabbit to split the lactose occurs as a possible explanation, although the work of Plimmer (1906) and the production of moderately severe hyperglycemias in several experiments would seem to demonstrate quite conclusively that lactases are present, and that this sugar is split by the rabbit organism. If, however, the sugar is split into glucose and galactose as such, a rise in blood sugar of more than 20 or 30 mg. could certainly be expected, especially since Cori(1925) has shown that absorption of these monosaccharides is essentially independent of their concentration in the intes-



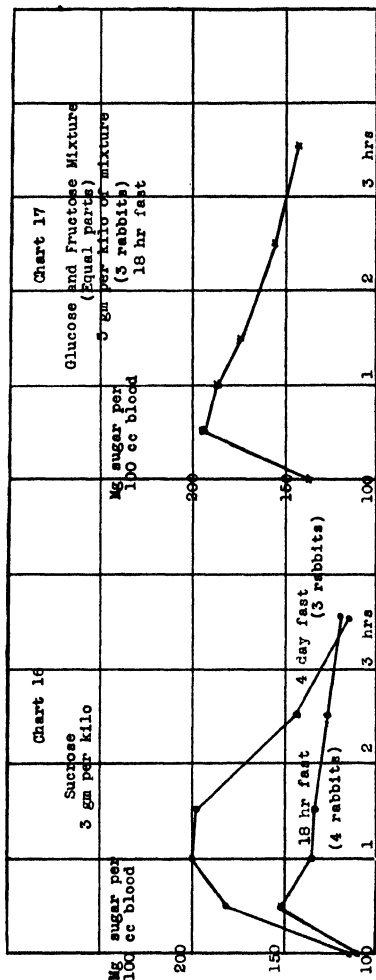
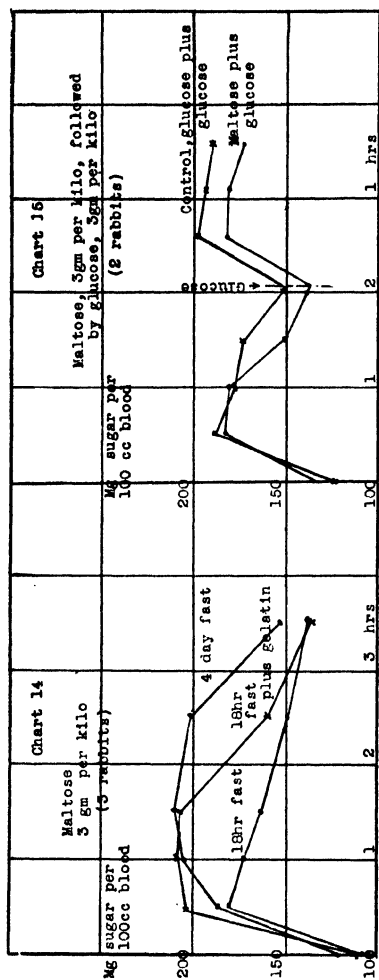
CHARTS 10 TO 13.

tine. It should also be remembered that galactose and glucose have the greatest hyperglycemia-producing ability of any of the sugars, which makes even more remarkable the failure of any decided hyperglycemia to appear following lactose ingestion. Practically the only explanation that would satisfy the conditions is that the substances produced by the fission of lactose by lactase are not chemically identical with those products obtained on acid hydrolysis *in vitro*, with the accompanying crystallization required to produce the crystalline forms fed in the experiments with simple sugars. Such a view is further supported by the failure of lactose to stimulate the glucose-removing mechanism as shown in the successive tolerances in which glucose followed lactose, the amount of glucose potentially available from the lactose fed being considerably in excess of the amount necessary to produce such stimulation. Further experiments in which lactase-hydrolyzed and acid-hydrolyzed lactose will be compared are planned for the near future, and may clarify somewhat this anomalous behavior of lactose.

### *Maltose.*

While the effect of maltose on blood sugar in general resembles that of glucose (Charts 14 and 15), it differs in one important respect, the effect of protein. In response to gelatin fed 18 hours previous to the sugar, maltose tolerance decreased, in this way differing from the other sugars studied, all of which showed increased tolerance under the same conditions. This behavior of maltose was without individual exception and of decided nature. Furthermore, it is in agreement with the behavior of maltose following starvation, this sugar showing less effect toward fasting than any other excepting lactose. Why maltose would behave in this unique manner is not apparent, but it emphasizes the existence of a definite interrelationship between protein and disappearance of sugars from the blood.

In this connection, the work of Compton (1924) is of interest in that he found the maltose of blood serum to be slightly more active during a fasting period than during digestion. Kumagai (1913) obtained a similar result. This may in part be the explanation of the relatively smaller starvation effect in maltose as compared with the other sugars, and fits in with the hypothesis, ad-



CHARTS 14 TO 17.

vanced by Staub (1922) that changes in enzyme activity are responsible for the fasting effect.

When maltose was followed by glucose, no appreciable rise was caused by the second sugar (Chart 15), thus demonstrating that maltose or its products have an ability equal to that of glucose to stimulate the glucose-removing mechanism.

### *Sucrose.*

Sucrose caused only a moderate hyperglycemia following administration by stomach tube (Chart 16). The curve reaches its maximum at the first half hour and drops gradually, and is essentially a composite of the glucose and fructose curves when these sugars are fed singly.

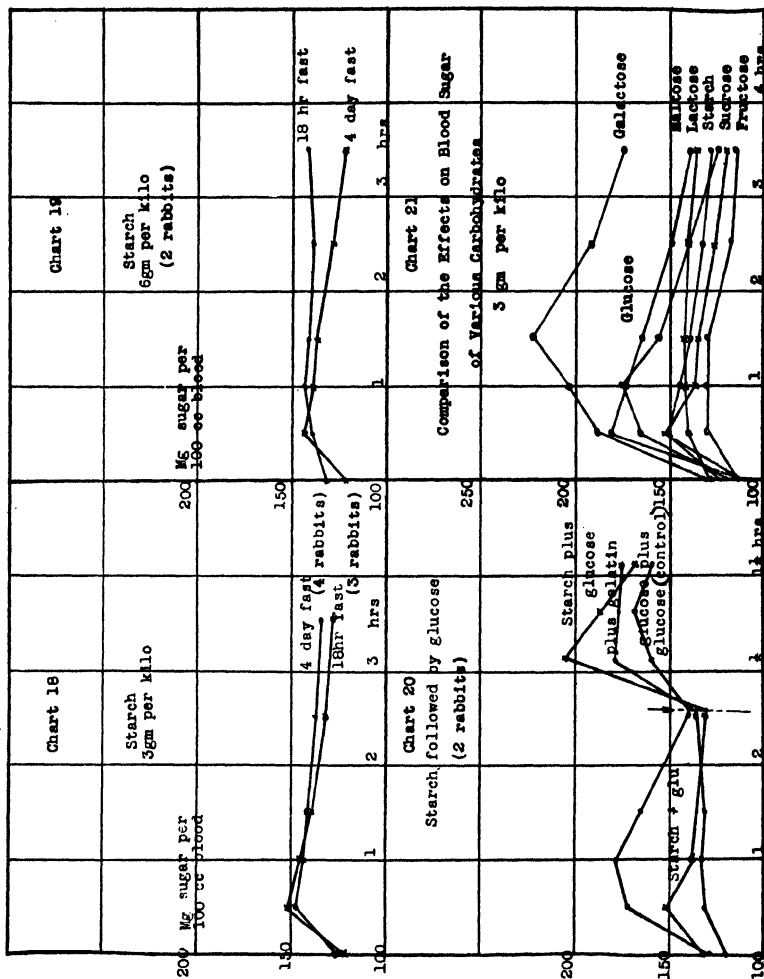
Fasting produced a marked lowering of the tolerance for this sugar, the shape of the fasting curves being similar to those of glucose under the same conditions.

Feeding a mixture of the component sugars of sucrose produced a somewhat greater rise than did sucrose (although less than 20 mg. higher), but in other respects the resulting curves were practically the same (Chart 17). The effect in this case, of feeding the two sugars is cumulative indicating that absorption of each is essentially independent of the other, although it is difficult to see why the effect of feeding sucrose should not be similar to that obtained with glucose-fructose mixtures. Here again the results seem to indicate a better utilization of the products of the enzymatic cleavage of a disaccharide than is obtained by the feeding of supposedly equivalent mixtures, and again no explanation is indicated. Possibly these effects are produced by impurities, although this seems unlikely, for the same sugars when fed individually produced no noticeable reaction, nor did the blood sugar curves following the ingestion of the sugars composing the mixtures singly, show any important abnormalities.

### *Starch.*

A moderate but very definite rise in blood sugar was produced by feeding starch through a stomach tube (Charts 18 and 19), the increase being unaffected by the variations in the quantity administered. Neither did fasting show any effect on the tolerance





CHARTS 18 TO 21.

comparable to behavior of other sugars, although there was a noticeable lowering of the tolerance following such treatment. Furthermore, starch failed to reduce the hyperglycemia produced by a successive dose of glucose (Chart 20). Since sufficient absorption occurred to produce a mild hyperglycemia, this failure to stimulate the glucose-removing mechanism may, as with lactose, indicate a difference between the glucose resulting from intestinal digestion of starch, and that resulting from ingestion of artificially prepared glucose.

Previous administration of gelatin resulted in increased tolerance for starch, while the rise following successive glucose ingestion was also reduced in those experiments in which gelatin was given. This, however, might be easily due to the beneficial effect of gelatin on glucose tolerance itself.

The relative ability of the carbohydrates studied to produce hyperglycemia may be seen in Chart 21.

#### DISCUSSION.

In the main (with exceptions mentioned below) the ability of the various carbohydrates studied to produce hyperglycemia appears to vary directly with their rates of absorption, but inversely with their ability to form glycogen and the ease with which they are oxidized in the animal. The interplay of these three factors seems sufficient to account for the differences observed in the behavior of the monosaccharides examined. Thus fructose besides having a slower absorption rate, has been shown by other workers to form glycogen most easily, and to be oxidized most readily. One would, accordingly expect under normal conditions only small increases in blood sugar, and this is confirmed by determinations of the tolerance for this sugar.

Following the ingestion of galactose the relative importance of the three factors is changed, for while galactose is rapidly absorbed, it is inferior in its ability to form glycogen and produces more moderate increases in heat production, and it consequently would be expected to produce a considerable increase in blood sugar during the latent period which is required to stimulate the mechanism involved in its removal. Glucose occupies an intermediate position in these respects.

It is difficult to explain the behavior of those carbohydrates

which following ingestion produce little or no hyperglycemia. Lactose, for example, shows only a maximum average rise of 12 mg. per 100 cc. of blood, yet it remains practically above the pre-ingestion level at the end of the  $3\frac{1}{2}$  hour period. Unless the absorption and utilization functions have reached an equilibrium so that the two processes may extend over a considerable period while remaining at or near the normal sugar level, it is necessary to assume that the products of such a digestion exist in that most readily utilizable form, or at least are capable of easy conversion into such forms. This is supported substantially by administration of the component sugars, singly and in mixtures.

Although it has been believed that the mature rabbit does not secrete a lactase, every rabbit used in the lactose experiments showed absorption, as reflected in increased blood sugar following ingestion of the lactose, which in a few cases (6 gm. per kilo, 4 day fast) reached the magnitude of hyperglycemias resulting from glucose ingestion. This group of experiments in which large doses of sugar were superimposed on fasting effects further shows that it was ease of utilization rather than slowness of absorption which was responsible for the absence of marked hyperglycemia.

With the exception of starch, the effect of inanition was to reduce the tolerance for carbohydrates, as shown by increased hyperglycemias of longer duration. There was, however, considerable variation among the different sugars which responded to the fasting effect, fructose, lactose, and sucrose showing the most decisive reactions, while galactose and maltose showed relatively moderate effects.

Gelatin, as is to be expected, produced the greatest effect on those sugars which were most influenced by fasting, and either no effect, or as in the case of maltose, an unfavorable effect on the tolerance for those sugars shown to be only slightly influenced by fasting.

Stimulation of the glucose-removing mechanism of the blood demonstrated by improved tolerance for a successive dose of glucose was observed for fructose, galactose, and maltose, but strangely, not for starch or lactose. The behavior of the monosaccharides may be caused as already pointed out, by a conversion either complete or partial into glucose or a common metabolite possessing the ability to stimulate its removal. Such a conversion

at least with galactose, is not very rapid as is shown by the tendency of this sugar when administered in successive doses to cause a rise following the second dose of sugar which is only slightly less than that following the initial feeding. On the other hand, the unchanged sugars may be responsible for their effect on a succeeding dose of glucose for it is quite conceivable that this stimulating effect may lie in a group common to the three monosaccharides studied. The fact that some time apparently must elapse before the stimulating effect becomes noticeable, however, would indicate that a preliminary change occurs, which may not, however, be essential to the stimulating action.

Lactose and starch, perhaps as a result of the production of readily utilizable substances lacking the stimulating group, may for that reason fail to affect this mechanism. Theoretically, maltose should behave similarly to starch and its failure to do so might easily be the result of a partial absorption of maltose, which as such may be able to influence the glucose-removing mechanism. On the other hand, differences in the products of enzyme action may furnish an explanation, the starch-splitting enzyme forming a different isomer than does intestinal or blood maltase.

As was mentioned previously, the failure of the component sugars of lactose and sucrose when fed as simulatory mixtures to produce blood sugar effects similar to those resulting from the disaccharides themselves cannot be accounted for on the basis of differences in absorption, nor can the agency which is responsible be detected. It seems possible that such discrepancies in the behavior of these sugars may be due to an activation produced during enzyme cleavage, or that the products of such cleavage are of a chemically different nature from those sugars obtained through the relatively drastic treatment of acid hydrolysis and crystallization. Impurities in the monosaccharides making up the mixtures may have accounted for some of the increased hyperglycemic action, although as previously pointed out, such influences did not seem particularly in evidence in experiments with single sugars, and if present, were apparently quite negligible in effect. It is to be hoped that a comparison of enzyme and acid hydrolysates will provide an explanation for this phase of sugar utilization, and further work is consequently planned in this direction.

## SUMMARY.

1. Galactose, maltose, sucrose, starch, lactose, and fructose were effective in the order named in producing alimentary hyperglycemia in rabbits.

2. A 4 day fasting period reduced the tolerance for all of the sugars studied. The effect was most pronounced with fructose, galactose, and sucrose. Lactose was only slightly affected and starch almost not at all.

3. Previous administration of protein (gelatin) reduced the hyperglycemic action of the carbohydrates studied with the exception of maltose, in which case the opposite effect was produced.

4. Maltose, galactose, and fructose were effective in reducing hyperglycemia resulting from a successive dose of glucose. Starch and lactose lacked this ability.

5. The hyperglycemias produced by mixtures of glucose and galactose and of glucose and fructose differ from those of the corresponding disaccharides, lactose and sucrose.

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## ON THIASINE, ITS STRUCTURE AND IDENTIFICATION WITH ERGOTHIONEINE.\*

BY ELEANOR B. NEWTON, STANLEY R. BENEDICT, AND  
H. D. DAKIN.

(From the Department of Chemistry, Cornell University Medical College, New  
York City.)

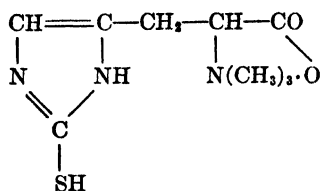
(Received for publication, December 6, 1926.)

The structure of thiasine, a sulfur-containing compound isolated from blood corpuscles and described by Benedict, Newton, and Behre (1), has recently been the subject of investigation and has led to some interesting results. In the paper just referred to the formula  $C_{12}H_{20}N_4O_3S$  was provisionally assigned to the compound which formed only a monohydrochloride and it was further shown that none of the nitrogen was in the amino form nor did the compound give any typical nitroprusside reaction. The fact that the compound while containing so much nitrogen behaved only as a mono-acid base suggested the possibility that the substance might be a betaine derivative of thiolglyoxaline. This idea received immediate confirmation from the fact that thiasine was found to give a typical Pauly reaction with sodium paradiazobenzenesulfonate, indicative of a glyoxaline ring, and that the behavior of its sulfur was closely parallel to that in thiolglyoxaline. Thus the sulfur in thiasine is remarkably stable towards alkali but is readily removed on oxidation with ferric chloride. Furthermore like other thiolglyoxalines thiasine quickly reduces cold dilute neutral potassium permanganate, while histidine and cystine do not react under comparable conditions. The presence of a betaine grouping in addition to the glyoxaline nucleus was rendered more probable by the fact that thiasine on warming with strong caustic potash readily evolves trimethylamine in amount equivalent to approximately 1 atom of nitrogen from each molecule of thiasine.

\* A preliminary announcement of the results of the work here reported appeared in *Science*, 1926, lxiv, 602, Dec. 17.



A consideration of the preceding observations naturally led to a comparison of thiasine with known thiolglyoxaline derivatives and it was soon clear that thiasine was identical with the base ergothioneine isolated from ergot in 1909 by Tanret (2), the constitution of which was determined by Barger and Ewins (3) 2 years later. Ergothioneine was shown to be the betaine of thiolhistidine and to have the following structure:



The elementary composition of thiasine as given by Benedict, Newton, and Behre together with the theoretical figures for ergothioneine are given below.

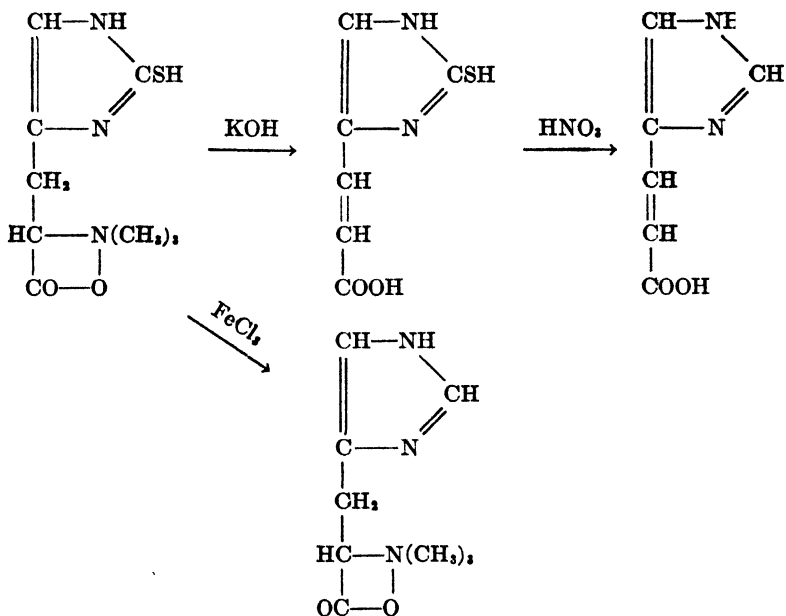
	Thiasine.	Ergothioneine.
Carbon.....	47.35	47.12
Nitrogen.....	18.20	18.33
Hydrogen.....	6.55	6.60
Sulfur.....	10.80	13.98

It will be noted that the figures for carbon, hydrogen, and nitrogen all show excellent agreement. The figure for sulfur is clearly too low and later experiments using preliminary oxidation with boiling neutral potassium permanganate gave the figures 10.31 for the hydrated hydrochloride (theory 10.62) and 13.40 for the free base (theory 13.98).

The specific rotation for ergothioneine is given by Tanret as  $+110^\circ$ , while Benedict, Newton, and Behre recorded  $+116^\circ$  for the compound from blood. Considering the high rotation of the compound, the agreement is quite as close as might be expected. The determination of rotation made by Benedict, Newton, and Behre was made using a 5 per cent solution of thiasine in a 2 dm. tube at  $21^\circ\text{C}$ . The melting point of ergothioneine is given by Tanret as  $290^\circ\text{C}$ . as observed with the "bloc Macquenne." As is well known this method for determining the melting point of substances melting at a high temperature

commonly gives values 20 to 30° higher than those obtained with the ordinary capillary tube method, so that Benedict, Newton, and Behre's figure of 262–263°C. (uncorrected) for their compound is tolerably close to Tanret's observation (4).

The simple and convincing reactions used by Barger and Ewins in elucidating the structure of ergothioneine were used without modification for the examination of thiasine and the two substances were found to behave in an identical fashion. Thus on boiling the base from blood with strong potash trimethylamine is evolved almost quantitatively and a yellow amorphous acid containing the whole of the sulfur is obtained from the residue. This substance, thioliminazolacrylic acid, on oxidation with dilute nitric acid gave urocanic acid, which was readily identified by means of its characteristic nitrate. On oxidizing thiasine with boiling ferric chloride solution, sulfur was removed from the ring with formation of histidine betaine. The changes may be represented as follows:



Ergothioneine was shown by Tanret to give addition compounds with iodine but the significance of the reaction was first appre-

ciated by Barger and Ewins, who found that the first action of iodine was to oxidize the base to a compound with two directly linked sulfur atoms (analogous to cystine formation from cysteine) and that this product had the property of taking up more iodine forming blue-black mixed crystals. The phenomenon is in many ways analogous to the adsorption of iodine by starch. It was found that the base derived from blood showed the reaction in typical fashion. It may be recalled that Benedict, Newton, and Behre showed that thiasine hydrochloride solutions behave in a peculiar fashion with silver nitrate. With moderately dilute solutions no precipitate forms but only a faint opalescence, and silver chloride is only precipitated after addition of nitric acid. It would seem not impossible that some physiological function may be correlated with these peculiar physical properties.

#### EXPERIMENTAL.

Thiasine (0.3 gm.) was slowly distilled with 10 cc. of 40 per cent aqueous potassium hydroxide. The alkaline vapors smelling of trimethylamine were collected in hydrochloric acid of which 5.5 cc. of  $N/5$  acid were neutralized. The acid distillate was concentrated and then precipitated with alcoholic platinic chloride. The salt obtained melted at 240–241°C. and contained 36.7 per cent platinum. The yield of trimethylamine was about 80 per cent of the theoretical.

On making the alkaline residue from the above distillation acid to Congo red with hydrochloric acid, a yellow amorphous acid (0.2 gm.) was precipitated. It was extremely insoluble in water and had all the properties of  $\beta$ -thiolglyoxaline-4-acrylic acid described by Barger and Ewins. It was oxidized by boiling for 3 minutes with 10 cc. of 10 per cent  $HNO_3$ . On cooling, a good yield was obtained of the characteristic bent sickle-shaped plates of urocanic acid nitrate. The salt was washed and dried. It melted with violent decomposition at 198–200°C. Free urocanic acid was obtained by adding 1 equivalent of sodium bicarbonate to the hot aqueous solution of the nitrate. Urocanic acid, melting at 230–233°C., crystallized readily and was identical with specimens of the acid obtained in other ways. The acid gave the characteristic picrate m.p. 212–214°C. and a typical Pauly reaction with diazo salts. It was further identified by

its nitrogen content 16.16 per cent; theoretical for urocanic acid 16.09 per cent.

Thiasine from blood was boiled for 1 hour with 7 parts of ferric chloride in 10 per cent solution. The product of the reaction was isolated precisely as described by Barger and Ewins and gave a moderate yield of the sparingly soluble dipicrate of trimethyl-histidine (histidine betaine). It melted at 121–123°C. when freshly crystallized from water, and when dehydrated, at 213–214°C. Its solubility and appearance as well as melting point were indistinguishable from the product obtained from ergothioneine.

Thiasine from blood treated with gold chloride yields a blood-red coloration as described by Tanret for ergothioneine. Thiasine also yields a green color with alkali and chloroform as described by Tanret for ergothioneine.

Ergothioneine was prepared from ergot<sup>1</sup> by the modified procedure of Georges Tanret (5) and obtained in the form of the pure hydrochloride. Simultaneous melting point determinations were made upon (1) a sample of this product, (2) a sample of thiasine hydrochloride prepared from blood, and (3) a mixture of these two samples. The contents of all three melting point tubes melted together sharply at 207–208°. The color yielded with arsenophosphotungstic acid in presence of cyanide by a weighed amount of the ergothioneine from ergot was compared with that from a similar quantity of the compound isolated from blood. The color yielded by the two samples was identical in intensity and shade.

Through the kindness of Dr. George Barger and M. Tanret we have recently come into possession of a fine crystalline specimen of ergothioneine hydrochloride prepared from ergot by M. Tanret. The melting point of this product, observed either alone or mixed with the hydrochloride of thiasine from blood, is identical with that of the latter taken alone (207–208°). It is a pleasure to express our appreciation of the generous cooperation shown us by both of the above mentioned workers.

#### CONCLUSION.

Thiasine has been identified as ergothioneine (betaine thiohistidine). Thus it would seem that the name thiasine should be

<sup>1</sup> We wish to express our appreciation to the Norwich Pharmacal Company of Norwich, New York, for their kind cooperation in supplying us with the ergot employed for preparation of ergothioneine.

abandoned. Yet the term ergothioneine seems hardly appropriate as applied to a product which is certainly widely distributed among mammals. We venture to suggest dropping the prefix "ergo" and using the term "thioneine." This would serve to indicate the sulfur content of the compound, as well as the fact that the sulfur is present in the SH form as in cysteine. It also retains the major portion of the name suggested by Tanret, the original discoverer of the compound in ergot.

It would be premature at the present time to speculate on the possible relationship of thioneine to other compounds in the organism or to suggest probable lines of its metabolism. The large amount of the compound present in corpuscles (we have isolated as much as 145 mg. per liter from whole pig blood) indicates that the compound is of very definite importance in the organism. Our observations up to the present time indicate that thioneine is present in human blood in amounts of from 10 to 25 mg. per 100 cc. of blood. We expect to report the isolation of thioneine from human blood in the near future.<sup>2</sup> We are continuing the study of the distribution of thioneine in the animal organism and of the fate of the compound in metabolism.

<sup>2</sup> While this paper was in press we have worked up 550 cc. of normal human blood for preparation of thioneine according to the method already detailed elsewhere (1). Preliminary color observations on this blood showed that it contained not more than 5 mg. of thioneine in 100 cc., which is perhaps the lowest thioneine content that we have ever observed in human blood. Nevertheless, from this blood we obtained a considerable quantity of the insoluble mercuric chloride salt. After decomposition of this salt with hydrogen sulfide and removal of the latter gas by boiling, the solution gave an intense reaction with phosphotungstic acid in presence of cyanide. On evaporation of the solution to a small volume and standing in the ice box numerous beautiful crystals separated which were identical in appearance with thioneine hydrochloride isolated from ergot and repeatedly isolated by us from pig blood. These crystals yielded very strongly the color reaction given by thioneine with phosphotungstic acid in presence of cyanide or carbonate. On account of the high solubility of thioneine and of its hydrochloride we were not able to recrystallize successfully the material from human blood for analysis. The appearance of the crystals, and the method of their preparation, together with the typical color reaction with phosphotungstic acid and alkali leave practically no doubt as to their identity with thioneine.

*Addendum.*—(Added at proof correction.) Our announcement in *Science*, December 17, of the identity of thiasine with ergothioneine was followed by the appearance of a paper by Eagles and Johnson (6) which was received by the *Journal of the American Chemical Society* 4 days after our publication had appeared, and which confirmed our finding of the identity of the uric acid-reacting compound in blood with ergothioneine. Eagles and Johnson make no mention of our announcement in *Science*, although their results are substantially similar to ours there reported. Eagles and Johnson have, however, brought out the fact that the compound described by Hunter and Eagles (7) as a levorotatory, sulfur-free compound, and which now, it appears, carries the name "sympectothion," is in reality identical with the uric acid-reacting compound first isolated from blood in this laboratory and which we have shown to be identical with ergothioneine.

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## THE NORMAL EXCRETION OF ZINC IN THE URINE AND FECES OF MAN.

BY KATHERINE R. DRINKER, J. W. FEHNEL, AND  
MARION MARSH.

*(From the Department of Physiology, Harvard School of Public Health,  
Boston, and the Palmerton Hospital, Palmerton, Pa.)*

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Studies upon the excretion of zinc in man have been largely concerned with the excretion of zinc compounds ingested, inhaled, subcutaneously or intravenously injected, and not with the excretion of the normal quota of zinc which enters the body daily through the medium of the food. It has been known for some years, though not widely recognized, that urine and feces normally contain zinc in by no means negligible quantities. Weitzel (1), in 1914, and later Rost and Weitzel (2, 3), writing together and separately, have reported the finding of zinc in normal human urine and feces. The approximate range of figures given by these authors as the usual normal was 3 to 19 mg. of zinc per day in feces, and 0.6 to 1.6 mg. in urine.

Batchelor, Fehnel, Thomson, and Drinker (4), 1926, in a clinical and laboratory study of the health of zinc workmen, included a table of control figures upon the amounts of zinc found in 24 hour samples of urine and of feces from normal individuals on an ordinary mixed diet. The range of figures reported in this paper is as follows: feces, 2.67 to 19.5 mg. of zinc per 24 hours; urine, 0.75 to 1.75 mg.

Fairhall (5) has very recently reported a series of figures upon the daily fecal zinc excretion of a man during a period of nearly 3 weeks. The general range of these figures, 5 to 19.7 mg., corresponds very closely to that reported by Batchelor and his associates and by Rost and Weitzel. An interesting peak, however, of 69.8 mg. on 1 day, with 36.3 mg. the following day, appears in Fairhall's fecal excretion curve as the result of having eaten oysters at a single meal. Oysters, it should be mentioned, have fre-



quently been shown to be unusually rich in zinc (Hiltner and Wichmann (6), Bodansky (7), Bertrand and Vladesco (8), etc.).

In the course of a somewhat extensive series of investigations being carried on in the Department of Physiology of the Harvard School of Public Health, upon the normal zinc metabolism and the significance of zinc to the living organism; upon the absorption, storage, and excretion of zinc; and upon the physiological and pathological action of zinc and its compounds upon the animal body, we have found it necessary to obtain a larger series of normal human zinc excretion figures than the few already reported in the literature, and have felt it important to determine to how great an extent the usual normal figures can be altered by the deliberate ingestion of foods with a high zinc content. Obviously, the finding of unusual amounts of zinc in the urine and feces of men exposed to zinc industrially has no very great diagnostic significance as evidence of poisoning, if the normal zinc content of urine and feces can be readily and markedly increased at will by changes in diet.

We wish to present in this paper, therefore, the results of the analyses of 50 24 hour specimens of urine and of feces from normal adults on an ordinary mixed diet, together with figures upon the urinary and fecal zinc excretion of two individuals for 8 consecutive days, upon 1 day of which food was especially selected with a view to increasing the zinc intake as much as possible.

#### EXPERIMENTAL.

*Collection of Specimens.*—Urine specimens were collected into carefully cleaned bottles; fecal specimens, into new, glazed pasteboard cartons. With two exceptions, the individuals supplying the 50 specimens included in Table I were nurses, orderlies, and laboratory workers at the Palmerton Hospital. The two individuals providing material for the weekly excretion data were laboratory workers in the Harvard School of Public Health.

*Diet.*—The diet of the individuals providing the 50 normal specimens (Table I) was an ordinary mixed diet. With the exception of the single high zinc meal, the diet of Subject 1 (Fig. 1) was also an ordinary mixed diet; that of Subject 2 was relatively high in protein, fruit, and roughage and was somewhat below the average of an ordinary mixed diet in carbohydrates and fats.

This was because Subject 2 was attempting to lose weight. An actual weight loss of  $3\frac{1}{2}$  pounds occurred in this subject during the experimental period. Her zinc excretion, however, previous to the high zinc meal was in the usual normal zone, indicating that the food ingested contained amounts of zinc within the usual range of an ordinary mixed diet.

The high zinc meal consumed by Subjects 1 and 2 in the middle of the experimental period was made up as follows: *Hors d'œuvres*, yeast cake with anchovies, chopped olives, and mayonnaise. Each subject ate approximately three-fourths of a yeast cake. Large raw oysters, twelve apiece. Oyster soup containing approximately eight oysters apiece. Duck, bread stuffing, apple sauce, wheat bran pudding, one large corn on the cob each, butter, (Subject 1, bread). Strawberry gelatin, whipped cream. Coffee and sugar.

The food materials in this meal especially rich in zinc were: yeast (414.8 mg. of zinc per kilo), oysters (26 to 2298 mg. per kilo), bran (139.2 mg. per kilo), corn (25.2 mg. per kilo), gelatin (27.4 mg. per kilo). These figures are taken from Lutz's (9) review of the normal occurrence of zinc in biological materials. An approximate estimate indicates that each subject ingested at this one meal between 225 and 275 mg. of zinc.

*Chemical Analysis.*—All specimens were dried, charred, and then ashed in an electric muffle furnace at 450°C. The residues were extracted with 1:1 distilled hydrochloric acid and hot water, filtered, and the filter paper and contents reashed and extracted. This was repeated as often as necessary for the complete destruction of all the organic matter.

Specimens were ashed and analyzed in their entirety. The acid ash solutions were treated with cupferron to remove iron. The zinc and added copper were then precipitated together in alkaline solution; the precipitate was well washed with water and with hot alcohol; it was then dissolved in nitric acid, the latter fumed off, and the copper removed in hydrochloric acid solution, leaving the zinc in pure form in the filtrate. The filtrate was then evaporated to dryness and made up to a known volume.

When the amount of zinc involved was in the range of 1 to 2 mg. or less (urines), the final solutions were read by the ferrocyanide turbidimetric method. When the amount was over 1 to 2 mg.

TABLE I.

*Zinc Content of Fifty 24 Hour Specimens of Urine and Feces from Twenty-Nine Healthy Adults on an Ordinary Mixed Diet.*

Subject No.	Sex.	Specimen No.	Urine.		Feces*	
			24 hr. volume.	Total sinc.	24 hr. quantity.	Total sinc.
			cc.	mg.	gm.	mg.
1	F.	1	1570	1.25	64	19.50
		2	1350	1.25	80	18.81
2	"	3	745	0.25	32	7.82
		4	1100	1.00	86	17.62
3	M.	5	1300	1.00	24	2.67
		6	1105	0.75	90	9.31
4	F.	7	1603	1.00	22†	2.75
		8	1805	1.38	24†	13.00
5	"	9	745	0.25	10	7.43
		10	820	0.75	85	13.36
6	"	11	1040	0.75	135	10.20
7	"	12	630	0.25	129	9.50
		13	1680	1.50	152	14.95
8	"	14	945	0.75	80	15.35
		15	1295	1.25	230	5.35
9	"	16	550	2.00	61	5.35
10	M.	17	2085	0.75	303	13.07
11	F.	18	1380	1.75	130	15.40
12	M.	19	1820	1.75	150	13.96
13	"	20	2600	1.50	140	14.36
		21	2230	1.25	170	7.13
14	"	22	2430	1.00	70	14.46
		23	840	0.50	180	5.54
15	"	24	1270	1.00	100	16.73
		25	950	0.25	110	13.66
16	"	26	1220	0.75	126	2.87
		27	2140	1.00	70	8.12
17	F.	28	1210	0.75	140	4.85
18	"	29	795	0.25	90	12.97
19	"	30	1470	1.25	40	3.66
20	"	31	940	1.25	130	10.40
		32	795	0.50	100	2.67
21	"	33	1230	0.25	75	10.89
		34	1210	1.00	87	5.15
22	"	35	1300	1.00		14.95
		36	940	0.75	70	6.73

\* With the exceptions noted, all fecal quantities are given as wet weights.

† Dry weight.

TABLE I—*Concluded.*

Subject No.	Sex.	Specimen No.	Urine.		Feces*	
			24 hr. volume.	Total zinc.	24 hr. quantity.	Total zinc.
			cc.	mg.	gm.	mg.
23	F.	37	590	0.50	40	9.50
		38	740	0.25	95	13.56
24	"	39	2170	0.75	210	4.75
		40	1830	1.00	38	3.37
25	"	41	1620	0.50	260	19.90
		42	1430	1.25	40	4.36
26	"	43	980	0.75	180	3.28
		44	1010	0.75	140	3.76
27	"	45	655	1.00	80	5.54
		46	795	0.25		8.81
28	"	47	1170	1.25	100	4.06
		48	850	0.50	35	6.53
29	M.	49	1190	0.70	39†	17.00
		50	1540	1.00	38†	14.80
Average . . . . .				0.89		9.80

(feces), zinc was determined by the ferrocyanide titration method. The foregoing procedure is outlined in detail by Fairhall (10).

### *Results.*

Table I contains complete data upon the quantity and the total zinc content of 50 24 hour specimens of urine and feces from twenty-nine healthy adults on an ordinary mixed diet. The range of figures is as follows: urine, 0.25 to 2 mg.; feces, 2.67 to 19.9 mg. The averages are: urine, 0.89 mg.; feces, 9.8 mg.

Complete data were obtained upon the daily output of zinc in the urine and feces of Subjects 1 and 2 for 8 consecutive days. Upon the 3rd and 4th days, the urine collections of Subject 2 were made in three lots for each day, the object of this procedure being to determine whether or not the daily excretion of zinc in urine goes on at a uniform rate.

3rd day.	Urine collections. cc.	Zinc content. mg.
8.30 a.m.-10.30 a.m.	600	0.44
10.30 " - 5.00 p.m.	625	0.20
5.00 p.m.- 8.30 a.m.	580	0.74
Total.....	1805	1.38
4th day.		
8.30 a.m.-1.30 p.m.	865	0.70
1.30 p.m.-5.00 "	310	0.66
5.00 " -8.30 a.m.	670	0.50
Total.....	1845	1.86

The results show conclusively that urinary zinc excretion varies greatly during the course of 24 hours, and they indicate clearly the importance of the analysis in all cases of a complete 24 hour specimen. In our opinion, figures for the total zinc content of urine for 24 hour periods are more consistent, more valuable, and more significant than are figures for the zinc content per cc. of urine, even when the latter are derived from analyses of 24 hour specimens.

In Fig. 1 is plotted the zinc excretion of Subjects 1 and 2 during the 8 experimental days. The significant facts to be observed in the curves of fecal excretion are:

(a) The very high zinc peak (198.25 mg.) reached in the curve of Subject 1. This figure represents the zinc in the fecal specimens collected during the 24 hours immediately following the high zinc meal, and is a higher figure than any reported by Batchelor and his associates (4) in the feces of their zinc workmen.

(b) The rapid fall of the curve—within 24 hours—from this high peak to a level (36.5 mg.) not far outside the usual normal zone (upper limit usually about 20 mg.), and the return upon the next day to a low normal. The rapid rise and rapid fall contrast with the slower rise and slower fall of the fecal excretion curve of Subject 1. Two causes are probably involved in this difference in the appearance of the two curves. The hour of the high zinc meal was 7 p.m. Subject 1's 24 hour collection periods began and ended at 6.30 p.m., whereas, those of Subject 2 began and ended at 8.30 a.m. The high point in Subject 1's curve represented, therefore, the zinc in feces passed during the first 23 hours after the high zinc

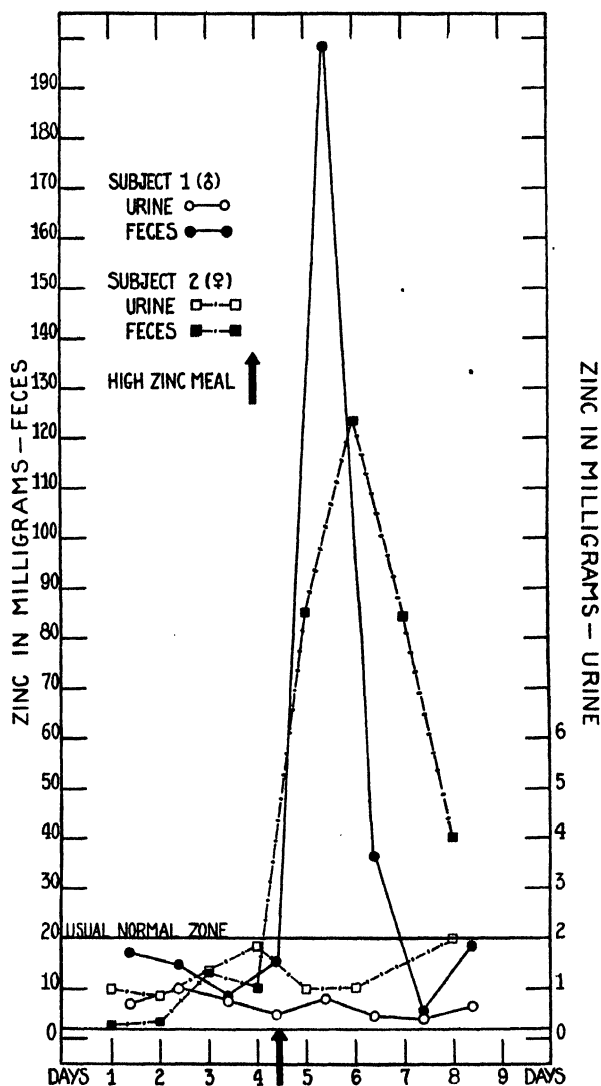


FIG. 1. Daily output of zinc in the urine and feces of two healthy adult subjects for 8 consecutive days. The arrow indicates the ingestion of a single meal made up of foods rich in zinc. Note the great increase in fecal zinc excretion resulting from this procedure.

meal, while the first point following the meal plotted for Subject 2 included only the first  $13\frac{1}{2}$  hours after the high zinc intake.

A second cause of the slower rise of Subject 2's curve and the probable explanation of its more gradual fall are the fact that Subject 2 is of a more or less constipated habit and rarely has more than one movement a day, while Subject 1 apparently hurries his food much more rapidly through the gastrointestinal tract and usually has two or three movements. On the day of the high peak in Subject 2's fecal curve, there were three movements for analysis.

The chief points of interest in the urine curves plotted in Fig. 1 are the great uniformity of normal urinary zinc excretion and the failure of the high zinc meal to cause a rise above the normal zone of 0.25 to 2.00 mg.

#### SUMMARY AND CONCLUSIONS.

1. Figures are presented upon the normal amounts of zinc present in the urine and feces of a large group of healthy adults on an ordinary mixed diet. The range and average of figures upon the total zinc content of 24 hour specimens is as follows: urine, range 0.25 to 2 mg., average 0.89 mg.; feces, range 2.67 to 19.9 mg., average 9.8 mg.

2. The normal daily total zinc content of the urine and feces of two healthy adults for 8 consecutive days is reported, and the effect upon the urinary and fecal zinc excretion of a high zinc meal is presented and discussed.

3. In addition to providing data for the establishment of normal standards of zinc excretion in man, the observations reported in this paper prove conclusively that amazing increases in fecal zinc excretion may occur following the ingestion of high zinc foods—increases which may last for several days. The work indicates also that significant increases in the normal content of zinc in urine do not result from the ingestion on single occasions of high zinc foods. The experiments presented, however, offer no evidence as to whether or not the normal urinary zinc level would be raised if foods unusually rich in zinc were continually ingested.

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## THE ACTION OF BLOOD ON SULFIDES.\*

BY W. DENIS AND LUCILLE REED.

*(From the Laboratory of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)*

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Although we are in possession of the records of a vast amount of experimental work on the pharmacological properties of hydrogen sulfide (1), the mode of oxidation of this substance by the blood and tissues is as yet not clear. Hydrogen sulfide is probably constantly present in the large intestine as a result of the bacterial decomposition of proteins. Lehmann (2) quotes analyses to show that hydrogen sulfide constitutes 1.0 per cent of the gases of the large intestine and Wells (3) reports the finding of 66 mg. of hydrogen sulfide per 100 gm. of colon contents. Furthermore the fact should be recognized that it is practically impossible to determine by experimental means the daily amount of hydrogen sulfide produced in the intestine on account of the very rapid absorption of this gas by the intestinal walls. Von Bunge (4) has called attention to the fact that, according to a series of determinations of the coefficients of absorption of the intestinal gases measured by Bunsen at 15°C., hydrogen sulfide has a coefficient of absorption of 3.2326 as against 0.02989 for oxygen; in other words, the former gas is absorbed 100 times more rapidly than is the latter. In view of the constant occurrence in the intestine, high coefficient of absorption, and intensely toxic properties of hydrogen sulfide, it is clear that, as under ordinary conditions no objectionable symptoms are noted, there must exist in the body some efficient mechanism for the detoxication of this substance. The nature of this detoxication process is, however, not definitely known.

By means of a series of ingenious experiments Haggard (5) has

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shown that blood plasma in the presence of oxygen possesses the property of rapidly oxidizing small amounts of hydrogen sulfide, and that the products of oxidation combine in part with the sodium of the plasma; whole blood also rapidly oxidizes hydrogen sulfide as a result of the withdrawal of oxygen from the corpuscles.

It seems reasonable to suppose that a considerable part of the hydrogen sulfide oxidized is transformed into  $\text{H}_2\text{SO}_4$ . Haggard has attempted to obtain proof of this transformation by measuring the  $\text{CO}_2$ -combining power of plasma before and after treatment with a known amount of  $\text{H}_2\text{S}$ , and has found that, while there was after treatment with  $\text{H}_2\text{S}$  an unmistakable fall in the  $\text{CO}_2$ -combining power of plasma, this drop amounted to from 20 to 30 per cent of the change which would have occurred had all of the  $\text{H}_2\text{S}$  oxidized been transformed into  $\text{H}_2\text{SO}_4$ . Obviously therefore it is necessary to consider the possibility of some type of reaction other than simple oxidation. Our knowledge of the sulfur-containing bodies of blood is exceedingly limited. We have recently published (6) a review of the work which has been done on the non-protein sulfur compounds of blood, from which it appears that the presence of inorganic sulfates, ethereal sulfates, and neutral sulfur in blood has been affirmed by some investigators, and denied in whole or in part by others. As the result of the analysis of a considerable number of samples of human and animal blood, we feel that the evidence now available would appear to indicate that the non-protein, non-lipoid sulfur of blood exists in the three forms in which sulfur is known to exist in urine; *i.e.*, as inorganic sulfate, in an unstable combination readily broken up by heating in acid solution, and probably analogous to the urinary ethereal sulfate, and as "neutral sulfur," determined by difference between total sulfur and total sulfates. As to what may be the nature of this neutral sulfur fraction of the blood, is a question which at the present time cannot be answered. In part at least it is possibly composed of cystine; whether two other sulfur compounds known to exist in blood, glutathione (7) and thiasine (8), may form a part of this neutral sulfur fraction is not known at present, although it seems to us highly probable that such may be the case.

## EXPERIMENTAL.

*Methods.*

In the experimental work described below an attempt has been made to obtain evidence regarding the nature of the oxidation products of hydrogen sulfide formed when this substance is introduced into the blood stream or placed in the intestine of the living animal. With this end in view samples of blood withdrawn at intervals after the administration of sulfides have been analyzed for inorganic and ethereal sulfates, and neutral sulfur by the gravimetric methods recently described (6).

As Haggard has shown that sodium sulfide on introduction into the blood stream is immediately hydrolyzed, this salt has in many of the experiments been employed in place of hydrogen sulfide on account of the fact that it can be more easily and accurately assayed than can the latter substance, thus affording greater precision in dosage.

All solutions of hydrogen sulfide and of sodium sulfide were standardized just before use by titration with 0.1 N iodine solution (9), using starch as indicator. As it was impossible to obtain solutions of sodium sulfide free from sulfate, sulfate determinations were made on all sulfide solutions used for injections. For this determination the method of Scott (10) was used. The amount of sulfate found was, however, so small (0.08 mg. of S per cc.) that it was disregarded in a consideration of the experimental results.

In the experiments described below 1.0 per cent sodium sulfide solution was injected into the external jugular vein of dogs at the rate of 1 cc. per minute, as suggested by Haggard (5). After each portion of sulfide was allowed to enter the vein, there was a marked increase in respiratory rate; this period of dyspnea lasted for approximately 30 seconds, and was followed by a period of apnea of approximately equal length. As a rule there was a return to the normal respiratory rate before the next injection was due.

*Experiment 1.*—January 4, 1926. Dog, male, weight 10 kilos. 60 mg. of morphine sulfate were injected subcutaneously. 1 hour later ether was administered and cannulas placed in the trachea, internal carotid artery, and external jugular vein; 200 cc. of blood were taken to serve as a control, and the intravenous

injection of sodium sulfide of 1.01 per cent concentration, started at the rate of 1 cc. per minute. After 28 cc. of this solution had been injected respiratory failure made it necessary to resort to artificial respiration, and at this time a second sample of 100 cc. of blood was removed from the carotid artery. Death from heart failure resulted 6 minutes later.

TABLE I.

*Experiment 1. Results Obtained Following Intravenous Injection of Sodium Sulfide.*

Sample.	Mg. S per 100 cc. blood.					Per cent on basis of total sulfur.		
	Inor- ganic SO <sub>4</sub> .	Ethe- real SO <sub>4</sub> .	Total SO <sub>4</sub> .	Neu- tral S.	Total S.	Inor- ganic SO <sub>4</sub> .	Ethe- real SO <sub>4</sub> .	Neu- tral S.
Before Na <sub>2</sub> S. ....	3.69	8.34	12.04	0.5	12.50	29.4	66.5	4.0
After " .....	3.69	9.74	13.4	0.5	13.90	27.5	72.2	3.6

TABLE II.

*Experiment 2. Results Obtained Following Intravenous Injection of Sodium Sulfide.*

Sample.	Mg. S per 100 cc. blood.					Per cent on basis of total sulfur.			
	Inor- ganic SO <sub>4</sub> .	Ethe- real SO <sub>4</sub> .	Total SO <sub>4</sub> .	Neu- tral S.	Total S.	Inor- ganic SO <sub>4</sub> .	Ethe- real SO <sub>4</sub> .	Total SO <sub>4</sub> .	Neu- tral S.
1. Before injection ....	2.91	3.99	6.90	3.62	10.53	27.7	38.0	65.7	34.4
2. After injection of 30 cc. Na <sub>2</sub> S. ....	2.41	3.13	5.54	4.42	9.97	24.1	31.5	55.5	44.3
3. After injection of 41 cc. Na <sub>2</sub> S. ....	2.41	3.19	5.60	5.49	11.09	21.7	28.8	50.5	49.5
4. After injection of 67 cc. Na <sub>2</sub> S. ....	4.03	3.24	7.27	3.32	10.59	38.0	30.6	68.6	31.3

The results of the analyses made on the blood of this animal are given in Table I.

*Experiment 2.*—February 8, 1926. Dog 4, male, weight 15 kilos. After anesthesia and cannulation as described under Experiment 1 a preliminary sample of 150 cc. of blood was taken from the carotid artery. Intravenous injection of a 1.09 per cent solution of sodium sulfide was then commenced at the rate of 1

cc. per minute. When 30 cc. had been injected, 100 cc. of blood were withdrawn (Sample 2), and after 11 cc. more a third sample. At this time a very distinct odor of hydrogen sulfide was noticed in the expired air, and lead acetate paper held near the tracheal cannula became dark immediately. After 26 cc. more of sodium sulfide had been administered a fourth and final 100 cc. portion of blood was withdrawn. 2 minutes later death occurred from heart failure. The total amount of sodium sulfide solution injected amounted to 67 cc. (equivalent to 275 mg. of S). The results of the analyses of the blood of this animal are given in Table II.

The results obtained in Experiment 1, and in several other experiments carried on along similar lines but which will not be reported in detail, are rather inconclusive, the increase in total sulfates and total sulfur being so small that it may fairly be said that they lie within the limits of error of the analytical methods used. That such results were obtained is not surprising when one considers the small amount of sulfide injected (28 cc. of 1 per cent solution) which is extremely small in proportion to the weight (10 kilos) of the animal.

In the next series of experiments of which Experiment 2 is given as a typical example greater care was exercised in timing the injections in order to keep the animal alive for a longer period; in Experiment 2 which lasted 78 minutes there was noted at the end of the experiment a moderate rise in the inorganic sulfates of the blood but no definite changes in the other fractions. In this experiment a loss of  $H_2S$ , the magnitude of which it is impossible to estimate, occurred by way of the expired air. In order to eliminate the possibility that this continued failure to show marked increase in the concentration of the various sulfur bodies might be due to rapid elimination by way of the kidney, two experiments were performed in which the blood supply of these organs was tied off.

*Experiment 3.*—December 17, 1925. Dog 3, female, weight 12.5 kilos. The experimental procedure was identical with that described for the two previous experiments except that the blood supply of both kidneys was tied off before the first sample of blood was taken. 19 cc. of 1.0 per cent  $Na_2S$  solution were then injected into the external jugular vein at the rate of 1 cc. per minute; at the end of this time a second sample of blood was taken. The dog died

of heart failure 7 minutes later. The results of the analyses of the blood are given in Table III.

*Experiment 4.*—January 6, 1926. Dog 4, male, weight 9 kilos. The experimental procedure was identical with that described for Experiment 3. 20 cc. of 1 per cent  $\text{Na}_2\text{S}$  (equivalent to 82 mg. of S) were injected intravenously at the rate of 1 cc. per minute, after which a second sample of blood was taken. The dog died 7 minutes later. The analytical results obtained on the blood are given in Table III.

TABLE III.

*Experiments 3 and 4. Results Obtained Following Intravenous Injection of  $\text{Na}_2\text{S}$  after Tying off the Kidneys.*

Blood sample.	Mg. S per 100 cc. blood.					Per cent on basis of total sulfur.			
	Inor-ganic $\text{SO}_4$ .	Ethe-real $\text{SO}_4$ .	Total $\text{SO}_4$ .	Neu-tral S.	Total S.	Inor-ganic $\text{SO}_4$ .	Ethe-real $\text{SO}_4$ .	Total $\text{SO}_4$ .	Neu-tral $\text{SO}_4$ .
Experiment 3.									
1. Before injection....	5.47	5.35	10.82	2.83	13.65	40.1	39.2	79.3	20.6
2. After injection of 19 cc. $\text{Na}_2\text{S}$ solution.	7.58	5.76	13.34	3.03	16.37	46.2	34.5	81.4	19.7
Experiment 4.									
1. Before injection....	5.20	5.48	10.69	2.40	13.10	39.7	41.9	81.6	18.3
2. After injection of 20 cc. $\text{Na}_2\text{S}$ .....	6.44	7.60	14.05	0.40	14.45	45.2	59.5	97.1	2.82

In Experiments 3 and 4 there is evidence of a decided increase in the concentration of inorganic and ethereal sulfates, but none in the neutral sulfur, and as the doses of sodium sulfide administered were comparatively small, the rise in sulfates is relatively great. The experimental results would suggest the importance of the kidney in the rapid removal of inorganic sulfates from the blood.

In order to study the effect of sulfide absorption from the intestine, sodium sulfide solution was injected into the duodenum of one animal and into the large intestine of another.

*Experiment 5.*—January 11, 1926. Dog 5, male, weight 10.2 kilos. Under morphine and ether anesthesia 100 cc. of blood were

removed from the carotid artery to serve as a control. The abdomen was then opened by an incision in the median line, and ligatures were placed around the small intestine just below the pylorus and above the ileocecal valve. 50 cc. of a solution of sodium sulfide saturated at room temperature with  $\text{H}_2\text{S}$ , which on analysis was found to contain 1.251 gm. of sulfide S in the amount injected, were then introduced into the duodenum.  $\frac{1}{2}$  minute later the respiratory rate showed a marked increase and subsided only after a period of 4 minutes. At this time (4 minutes after injection)

TABLE IV.

*Results Obtained Following Introduction of Sulfide in the Intestine.*

Blood sample.	Mg. S per 100 cc. blood.					Per cent on basis of total sulfur.			
	Inorganic $\text{SO}_4$ .	Ethereal $\text{SO}_4$ .	Total $\text{SO}_4$ .	Neutral S.	Total S.	Inorganic $\text{SO}_4$ .	Ethereal $\text{SO}_4$ .	Total $\text{SO}_4$ .	Neutral $\text{SO}_4$ .
Experiment 5. Absorption by duodenum.									
1	2.02	4.59	6.61	3.47	10.08	20.0	45.5	65.5	34.4
2	3.27	1.65	5.22						
3	3.19	3.81	7.00	2.24	9.74	32.7	39.0	71.8	28.1
Experiment 6. Absorption by large intestine.									
1	3.64	4.65	8.29	3.58	11.87	30.6	39.1	69.8	30.1
2	5.26	3.70	8.96	2.58	11.54	45.6	32.0	77.6	22.3
3	5.43	3.86	9.30	2.46	11.76	46.2	32.4	79.0	21.0
4	5.43	2.8	8.25	1.96	10.19	53.3	27.5	80.8	19.3

a strong sulfide test with lead acetate paper was given by the expired air, and continued during the remainder of the experiment. 6 minutes after injection a second sample of blood was withdrawn, and 14 minutes later a final 100 cc. portion of blood was taken. 3 minutes after this last withdrawal of blood the heart stopped beating.

*Experiment 6. Absorption from Large Intestine.*—January 12, 1926. Dog 10, male, weight 17 kilos. In this experiment the procedure was identical with that described for Experiment 5 except that a 2.2 per cent solution of  $\text{Na}_2\text{S}$  saturated with  $\text{H}_2\text{S}$  gas (which on analysis was calculated to contain 4.4 per cent  $\text{H}_2\text{S}$ ) was injected into the large intestine 15 cm. below the ileocecal



valve. A ligature placed around the intestine just below the cecum prevented the injected solution from entering the small intestine. After removal of the first sample of blood 25 cc. of the sulfide solution described above were injected into the intestine. There was no rise in the respiratory rate or the appearance of  $\text{H}_2\text{S}$  in the breath during a period of 15 minutes, and a further injection of 25 cc. of the solution was therefore made. 2 minutes after this second injection the respiratory rate was notably increased and  $\text{H}_2\text{S}$  was noted in the expired air. 3 minutes after the second injection a second sample of blood was taken, 28 minutes later a third, and after 47 minutes a fourth portion of blood was withdrawn.

The results obtained on the analysis of the blood taken in Experiments 5 and 6 are presented in Table IV, and here, as in the experiment in which sulfide was injected directly into the blood stream, there was an increase only in the inorganic sulfate fraction.

In these two experiments a comparison of the rates of absorption of sulfide in the large and in the small intestine may explain why hydrogen sulfide in the large intestine of a normal individual causes no discomfort. If hydrogen sulfide were found in any quantity in the small intestine, we would expect unpleasant evidence of its presence.

As a whole the experiments reported above would seem to indicate that sulfides introduced into the blood stream or absorbed from the intestine are oxidized to sulfuric acid. If the injection, or the absorption from the intestine, is rapid, the amount of sulfide present in the blood may be so great that it cannot be oxidized at once, in which case an unknown proportion of the absorbed  $\text{H}_2\text{S}$  may be eliminated through the lungs.

Titration by means of 0.10 N HCl of several lots of the 1.0 per cent sodium sulfide solutions used showed an alkalinity of  $\pm 0.19$  N. The injection of these solutions would introduce considerable quantities of alkali into the blood, and theoretically at least one would expect to find an increase in the alkali reserve. Haggard (11) has however found that there is a lowering of the alkali reserve following the injection of sodium sulfide solutions into etherized dogs. This decrease he has attributed to the condition of acapnia which occurred in his experiments during which alkali passed out of the blood and into the tissues in proportion to

the decreased carbon dioxide content of the blood. We have determined the alkali reserve (12) of the blood of three dogs under ether anesthesia before and after the injection of sodium sulfide solutions. The results of these analyses are presented below and offer confirmation of the results of Haggard. They indicate that in spite of the injection of strongly alkaline sodium sulfide solutions the level of the alkali reserve of our animals was that characteristic of an acidosis. We therefore feel that the changes in the level of the inorganic and ethereal fractions of the blood found in some of our experiments cannot be attributed to an alkalosis brought about by the alkaline sulfide solutions injected.

*Results of Determinations of the Alkali Reserve of the Blood of Etherized Dogs before and after the Administration of Sodium Sulfide Solutions.*

	Alkali reserve, vols. per cent CO <sub>2</sub>
Dog 2. Under deep ether anesthesia . . . . .	31.9
32 minutes later after injection of 30 cc. of 1 per cent Na <sub>2</sub> S solution . . . . .	27.1
Dog 3. Under deep ether anesthesia . . . . .	31.9
20 minutes later after injection of 19 cc. of 1 per cent Na <sub>2</sub> S solution . . . . .	30.0
Dog 4. Under deep ether anesthesia . . . . .	27.1
22 minutes later after injection of 20 cc. of 1 per cent Na <sub>2</sub> S solution . . . . .	23.3

SUMMARY.

Analysis of the blood of dogs who had received sodium sulfide by intravenous injection, or by injection into the intestine, sometimes showed a rise in the inorganic sulfate but no increase in the neutral sulfur fraction. These increased values were noted particularly in the case of animals in whom the kidneys had been tied off. The results are believed to give support to the theory that hydrogen sulfide is detoxicated by oxidation to sulfuric acid.

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## THE CHEMICAL STUDIES OF THE OVARY.

### XII. THE FATTY ACIDS OF THE LECITHIN FROM CORPUS LUTEUM.

BY MERRILL C. HART AND FREDERICK W. HEYL.

(From the Research Laboratories, The Upjohn Company, Kalamazoo.)

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The term lecithin as used today signifies a mixture of individual lecithins. This new view-point has been brought about by Levene and others who have shown that well known lecithins, from the brain, liver, and egg yolk, give on hydrolysis usually about five different fatty acids. This coupled with the fact that Levene<sup>1</sup> has shown that lecithins exist in monomolecular form necessitates our present conception of the term. The nature of the fatty acids incorporated in the lecithin molecule may also vary as McCollum, Halpin, and Drescher<sup>2</sup> showed in the case of the phosphatides of the egg yolk, the fatty acids of which they demonstrated varied with the diet. We have been interested in the theory of the non-specificity of the lecithins of corpus luteum and have considered that their main physiological importance was probably the formation of a more labile fat metabolism. With the rapid cell multiplication during pregnancy there is a self-evident necessity for an adequate supply of lipid material for cell structure. That there is an increased metabolism of fat during the period of pregnancy is indicated by the work of Tyler and Underhill,<sup>3</sup> Bloor,<sup>4</sup> Slemons,<sup>5</sup> and others, who have shown that the blood at this time is more effective as a carrier of lipid material. During the greater part of the period of pregnancy it is

<sup>1</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, *xlvi*, 185.

<sup>2</sup> McCollum, E. V., Halpin, J. G., and Drescher, A. H., *J. Biol. Chem.*, 1912-13, *xiii*, 219.

<sup>3</sup> Tyler, M., and Underhill, F. P., *J. Biol. Chem.*, 1925, *lxvi*, 1.

<sup>4</sup> Bloor, W. R., *J. Biol. Chem.*, 1921, *xlix*, 201.

<sup>5</sup> Slemons, J. M., and Stander, H. J., *Bull. Johns Hopkins Hosp.*, 1923, *xxxiv*, 7.

our opinion that it is one of the important functions of the corpus luteum to furnish an adequate supply of this more labile lipoid material for distribution by the blood. If this more general action of the phosphatides is true, we should expect that on hydrolysis of the lecithins from corpus luteum we would obtain in general the same acids that have been found by us<sup>6</sup> to be present in the neutral fat.

In Table I we have compared the fatty acids found on the hydrolysis of the lecithin from the acetone extract of corpus luteum with the fatty acids present in the neutral fat. We also have

TABLE I.

*Comparison of Fatty Acids of Lecithins from Acetone and Ether Extract of Corpus Luteum with Fatty Acids of Neutral Fat.*

Fatty acids.	Neutral fat.	Lecithin (acetone extract).	Lecithin (ether extract).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Saturated.....	35	43	48
Unsaturated.....	65	57	52
Palmitic.....	25	17	48*
Stearic.....	12	26	
Oleic.....	32	22	40
Linolic.....	16.6	26	8†
Arachidonic.....	8	7	2
C <sub>30</sub> H <sub>58</sub> O <sub>2</sub> .....	4.8	2	2
C <sub>16</sub> H <sub>30</sub> O <sub>2</sub> .....	1.6		

\* Probably a mixture of palmitic and stearic acids. See foot-note 7.

† All petroleum ether-insoluble bromides calculated as linolic tetrabromide.

incorporated the results obtained on the hydrolysis of a small amount (9.78 gm.) of the lecithin from the ether extract of corpus luteum.<sup>7</sup> We have made an approximation of the various percentages of the different fatty acids present.

We interpret the above results to signify that the physiological importance of the phosphatides of the corpus luteum is non-specific and depends on the production of a more labile fat mechanism.

<sup>6</sup> Cartland, G. F., and Hart, M. C., *J. Biol. Chem.*, 1925, lxi, 619.

<sup>7</sup> Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1926, lxx, 663.

## EXPERIMENTAL.

The material studied consisted of 38.6 gm. of purified lecithin cadmium chloride salt from the acetone extract of corpus luteum. This had been purified by the method outlined by us in a previous paper.<sup>8</sup> It contained 2.98 per cent of phosphorus and 1.38 per cent of nitrogen (N:P::1.026:1). This lecithin preparation was practically free from cephalin contamination as only 0.8 per cent of the total nitrogen was in the amino form.

37.271 gm. of this pure lecithin cadmium chloride compound were hydrolyzed by boiling under a reflux with 500 cc. of 10 per cent hydrochloric acid for 16 hours. The reaction mixture was cooled and the fatty acids extracted with ether. The washed and dried ether extract yielded on evaporation 18.412 gm. (49.4 per cent) of fatty acids.

The fatty acids were distilled at 160–215°C. at 0.6 mm. of mercury pressure. The distilled acids on cooling formed a white solid that weighed 15.217 gm. This material was separated by the barium-lead salt process of Levene<sup>9</sup> into a saturated acid plus oleic fraction and a fraction containing the more highly unsaturated acids. The oleic fraction was separated from the solid acids by the solubility of its lead salt in ether, by the Gusserow-Varrentrapp method.

By this means the fatty acids were separated into the following three fractions:

1. 6.138 gm. of saturated acids.
2. 2.290 " " intermediate fraction of the unsaturated acids (oleic).
3. 5.857 " " the more highly unsaturated acids.

*Examination of the Saturated Acids.*

This fraction was studied by systematic crystallization from alcohol and fractional precipitation and crystallization of the magnesium salts from alcohol solution. The magnesium salts were separately decomposed with dilute hydrochloric acid and the resulting acids crystallized further from alcohol. Three main fractions were obtained.

*Fraction 1.*—Weight, 0.225 gm. This melted at 67–68°C. It

<sup>8</sup> Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1925, lxxvi, 639.

<sup>9</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

was dried to constant weight *in vacuo* at the temperature of boiling toluene and analyzed.

*Analysis.*

0.1527 gm. substance:  $\text{CO}_2$  0.4237,  $\text{H}_2\text{O}$  0.1709.

Calculated for  $\text{C}_{18}\text{H}_{34}\text{O}_2$ . C 76.0, H 12.7.

Found. " 75.7, " 12.5.

A mixed melting point with pure stearic was not depressed. The acid in this fraction is stearic.

*Fraction 2.*—Weight, 0.504 gm. This was dried in the usual manner and analyzed.

*Analysis.*

0.1503 gm. substance:  $\text{CO}_2$  0.4154,  $\text{H}_2\text{O}$  0.1713.

Calculated for  $\text{C}_{16}\text{H}_{32}\text{O}_2$ . C 75.0, H 12.5.

" "  $\text{C}_{18}\text{H}_{34}\text{O}_2$ . " 76.0, " 12.7.

Found. " 75.4, " 12.8.

This fraction melted at 56–57°C. The melting point and analysis of this fraction indicate a mixture of palmitic and stearic acids.

*Fraction 3.*—Weight, 0.310 gm. This material represents the most soluble fraction of the saturated acids. It was dried and analyzed in the usual manner.

*Analysis.*

0.1568 gm. substance:  $\text{CO}_2$  0.4329,  $\text{H}_2\text{O}$  0.1770.

Calculated for  $\text{C}_{16}\text{H}_{32}\text{O}_2$ . C 75.0, H 12.5.

" "  $\text{C}_{18}\text{H}_{34}\text{O}_2$ . " 76.0, " 12.7.

Found. " 75.3, " 12.6.

This fraction melted at 56–57°C. The results indicate a mixture of palmitic and stearic acids.

*Examination of the Intermediate Fraction (Oleic).*

This fraction weighed 2.290 gm. and had an iodine number of 124.2. As this fraction contained acids more unsaturated than oleic, it was brominated in cold ether solution by the method of Baughman and Jamieson.<sup>10</sup> The ether-insoluble bromides weighed 0.3174 gm. These decomposed at 218°C. They were separated

<sup>10</sup> Baughman, W. F., and Jamieson, G. S., *J. Am. Chem. Soc.*, 1922, liv, 2947.

by extraction with boiling benzene in a Soxhlet apparatus into a benzene-insoluble and benzene-soluble fraction. The benzene-insoluble fraction weighed 0.113 gm. and decomposed at 233–234°C. 0.091 gm. of benzene-soluble material separated out on the cooling of the hot benzene extracts. These fractions were examined in conjunction with similar fractions isolated from the bromination of the more highly unsaturated acids.

The ether filtrate from the separation of the ether-insoluble bromides was washed with sodium thiosulfate solution until free from excess bromine, washed with water, dried, and the ether removed. The colorless oil obtained on dilution with 10 cc. of cold petrolic ether gave 0.261 gm. of white insoluble material. This on crystallization from petrolic ether gave 0.166 gm. of white crystalline tetrabromide melting at 110–111°C. This was examined in conjunction with similar material isolated from the bromination of the more highly unsaturated acids.

The petrolic ether filtrate and washings from the tetrabromide fraction described above contained 2.894 gm. of an oily material. The bromine was removed from this fraction by the method of Wesson.<sup>11</sup> 0.875 gm. of an oil with an iodine number of 111 was obtained. This was examined in conjunction with a similar fraction isolated in the later bromination.

#### *Examination of the More Highly Unsaturated Acids.*

The iodine number of this fraction was 175. On bromination<sup>10</sup> it gave 2.492 gm. of ether-insoluble bromides. These decomposed at 228°C. The benzene-insoluble fraction of these ether-insoluble bromides weighed 1.093 gm. and decomposed at 234–235°C. The 0.113 gm. of similar material obtained on the previous bromination was added to this. A sample of this material was dried to constant weight at the temperature of boiling toluene and analyzed for bromine by the Carius method.

#### *Analysis.*

0.1493 gm. substance: AgBr 0.2385.

Calculated for  $C_{20}H_{12}O_7Br_8$ . Br 67.8.

Found. " 68.0.

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<sup>11</sup> Wesson, L. G., *J. Biol. Chem.*, 1924, lx, 183.



This material is arachidonic octobromide and it results from the bromination of the arachidonic acid present in the lecithin.

0.012 gm. of material (decomposition point 228°C.) was insoluble in the hot benzene extract. The benzene extract separated on cooling 0.628 gm. of white material decomposing at 226°C. This on crystallization from hot benzene gave 0.450 gm. of material decomposing at 226°C. This material was dried to constant weight in the usual manner and analyzed.

*Analysis.*

0.1524 gm. substance: AgBr 0.2329.

Calculated for  $C_{20}H_{32}O_2Br_8$ . Br 67.8.

" "  $C_{20}H_{34}O_2Br_8$ . " 61.0.

Found. " 65.0.

The absence of any suggestion of material in this fraction melting at 183°C. shows the absence of linolenic hexabromide. The analysis of this fraction agrees fairly closely for a mixture of 60 per cent of arachidonic octobromide and 40 per cent of a hexabromide of the  $C_{20}$  series. We first found indications for this benzene-soluble hexabromide in the neutral fat of corpus luteum.<sup>6</sup> In this work the bromine was removed, the unsaturated acid reduced with hydrogen, and the solid acid crystallized and analyzed. Arachidic acid was found, indicating that this acid belonged to the  $C_{20}$  series. From the acids obtained from the hydrolysis of the lecithin from the ether extract of corpus luteum<sup>7</sup> we also obtained an ether-insoluble, benzene-soluble bromide fraction. This analyzed for 60.3 per cent bromine and checks fairly closely for the calculated bromine in this hexabromide. Evidence of this acid has also been found by us in the fat of ovarian residue.<sup>12</sup> It is interesting to note in this connection that Eckstein<sup>13</sup> found the benzene-soluble fraction of the ether-insoluble bromides from the subcutaneous fat of man to contain 61.0 per cent of bromine, which checks exactly for this hexabromide of the  $C_{20}$  series. We suggest ovarenic acid as a name for this new tri unsaturated acid of the  $C_{20}$  series.

The ether filtrate from the insoluble bromides was freed from excess bromine by washing with sodium thiosulfate solution,

<sup>12</sup> Tourtellotte, D., and Hart, M. C., *J. Biol. Chem.*, 1926-27, lxxi, 1.

<sup>13</sup> Eckstein, H. C., *J. Biol. Chem.*, 1925, lxiv, 797.

washed, dried, and the ether removed. The oily residue was diluted with 10 cc. of petroleum ether and on standing in the cold some white insoluble material separated. This insoluble material was centrifuged off, washed twice with petroleic ether, and dried.

This petroleic ether-insoluble material was dissolved in 8 cc. of ethyl ether and held at 0°C. for several hours. The solution was centrifuged to remove traces of ether-insoluble material and the filtrate evaporated to dryness. Weight, 1.430 gm. This was crystallized into two fractions from a mixture of ether and petroleic ether. The top fraction consisted of an oily material that was filtered off. The lower fraction on crystallization from petroleic ether gave 0.330 gm. of material melting at 109–110°C. To this was added a similar fraction from the previous bromination. This material was then split into five fractions by crystallization from petroleic ether. The top and bottom fraction melted at 112–113°C. All five fractions were united. Weight, 0.227 gm. This was dried to constant weight at the temperature of boiling toluene and analyzed.

*Analysis.*

0.1700 gm. substance: AgBr 0.2179.

Calculated for  $C_{18}H_{32}O_2Br_4$ . Br 53.3.

Found. " 54.5.

These analytical results indicate that the petroleum ether-insoluble bromide is largely linolic tetrabromide.

The petroleic ether filtrate from the tetrabromide fraction contained 6.387 gm. of material. The bromine was removed from this by the method of Wesson.<sup>11</sup> 1.856 gm. of an oil with an iodine number of 142 were obtained. This material evidently consisted of oleic acid contaminated with some of the more highly unsaturated acids. To get more information as to whether oleic acid was present or not, this fraction plus a similar fraction from the previous bromination, was oxidized. The method given by Lewkowitsch<sup>14</sup> was followed. The water-insoluble oxidation products were extracted with ether. This ether extract on concentration gave crystals melting at 127°C. These crystals

<sup>14</sup> Lewkowitsch, J., *Chemical technology and analysis of oils, fats and waxes*, London, 6th edition, 1921, i, 575.

were further purified by being extracted thrice with boiling water and the residue crystallized from alcohol. 0.0988 gm. of crystals melting at 130–131°C. was obtained. These were dried to constant weight at the temperature of boiling toluene and analyzed.

*Analysis.*

0.0964 gm. substance: CO<sub>2</sub> 0.2404, H<sub>2</sub>O 0.0984.

Calculated for C<sub>18</sub>H<sub>32</sub>O<sub>4</sub>. C 68.3, H 11.4.

Found. " 68.0, " 11.4.

A mixed melting point with dihydroxystearic acid was not depressed. This acid is dihydroxystearic acid, indicating the presence of oleic acid in this fraction.

The residue from the ether extract on extraction with hot water gave 0.357 gm. of material melting at 154–156°C. This on crystallization from 2 cc. of 70 per cent alcohol gave crystals of sativic acid melting at 156–157°C. This is further proof of the presence of linolic acid in the mixture of fatty acids studied.

SUMMARY.

1. The total fatty acids from the hydrolysis of the lecithin cadmium chloride compound from the acetone extract of corpus luteum amounted to 49.4 per cent. Of these 43 per cent were saturated and 57 per cent unsaturated. The experimental results obtained indicate the following approximate composition of the fatty acid mixture: 17 per cent palmitic, 26 per cent stearic, 22 per cent oleic, 26 per cent linolic, 2 per cent ovarenic (C<sub>20</sub>H<sub>34</sub>O<sub>2</sub>), and 7 per cent of arachidonic.

2. The fatty acids incorporated in the lecithin molecule from corpus luteum are similar to those found in the neutral fat. This finding is explained on the assumption that the physiological function of these phosphatides is a general one and consists in increasing the lability of the lipoids for which there is an increased demand during the period of pregnancy.

## ON THE SEPARATION OF HISTIDINE AND ARGININE.

### II. THE SEPARATION OF THE SILVER COMPOUNDS AT pH 7.0.\*

BY HUBERT BRADFORD VICKERY AND CHARLES S.  
LEAVENWORTH.

*(From the Laboratory of the Connecticut Agricultural Experiment Station,  
New Haven.)*

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In 1900 Kossel and Kutscher (1) showed that when barium hydroxide was added to a solution containing histidine and arginine together with an excess of a soluble silver salt, histidine silver was first precipitated completely and arginine silver began to precipitate only after the addition of a "somewhat greater excess" of the alkali. A few years ago Kossel and Edlbacher (2) suggested that the silver compound of histidine is completely precipitated at a faint alkaline reaction to phenolphthalein but that the silver compound of arginine does not come down until a still more alkaline reaction is reached. They imply that a separation may thereby be effected. We recently (3) had occasion to examine the behavior of these silver compounds and found that at the reaction represented by the first faint pink of phenolphthalein, all of the histidine together with a large part of the arginine was precipitated. We drew attention, however, to the possibility that a separation of these bases might be effected if the hydrogen ion concentration at which the precipitation of histidine silver is complete were ascertained.

This has now been found to lie close to pH 7.0. A practically complete separation of these bases can be readily obtained by bringing the solution, containing an excess of a soluble silver salt, to this reaction by the careful addition of barium hydroxide.

Preliminary experiments were carried out with solutions of pure

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

histidine and arginine in very dilute sulfuric acid. To a series of solutions of histidine sulfate, containing an excess of silver sulfate,

TABLE I.

*Data of Experiments in Which Histidine Silver Was Removed at Different Hydrogen Ion Concentrations and Arginine Silver Subsequently Precipitated.*

In Experiments 1 to 4 a single precipitation and in Nos. 5 and 6 a double precipitation of the silver compound was employed.

Experiment No.	Reaction.	Histidine N taken.	Arginine N taken.	N in histidine fraction.	Arginine N* in histidine fraction.	N in arginine fraction.	Histidine N in arginine fraction.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	<i>pH</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	7.2	0.158	0.357	0.165	0.014	0.343	
2	7.0	0.210	0.357	0.216	0.017	0.345	Trace.
3	6.8	0.237	0.469	0.243	0.019	0.435	"
4	6.6	0.207	0.488	0.168	0.028	0.504	0.0094†
5	7.0	0.206	0.485	0.195	0.013	0.468	
6	7.0	0.232	0.447	0.233	0.007	0.447	

\* The data in Experiments 3 to 6 were obtained by Kochler's modification of the Van Slyke method and are corrected for the ammonia set free by the partial decomposition of histidine.

† Owing to the accidental loss of a part of the histidine mercury precipitate this figure represents only a fraction, probably about one-half, of the histidine actually present in this solution.

TABLE II.

*Data Showing Recovery of Arginine and Histidine as Salts of Dinitronaphthol-sulfonic Acid.*

Experiment No.	Histidine N in di-salt.	Recovery based on N of histidine fraction.	Recovery based on histidine N taken.	Arginine N in mono-salt.	Recovery based on N of arginine fraction.	Recovery based on arginine N taken.
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
1	0.143	87.0	90.6	0.324	94.3	90.7
2	0.204	94.6	97.1	0.308	88.9	84.3
3	0.212	87.2	89.5	0.395	90.6	84.1
5	0.184	94.4	89.4	0.449	95.9	92.6
6	0.228	97.8	98.2	0.427	95.6	95.6

increasing quantities of dilute barium hydroxide solution were added, the precipitates centrifuged off, and their nitrogen content

determined. In this way the conditions under which all of the histidine present was precipitated were readily found. The hydrogen ion concentration of the filtrates from the histidine silver was estimated colorimetrically<sup>1</sup> by comparison with standard buffer solutions. The reaction of the solution from which the histidine had been completely precipitated and which contained the least quantity of barium hydroxide was in the vicinity of pH 7.2. Similar experiments in which dilute barium hydroxide was added to a solution of arginine and an excess of silver sulfate, showed that no nitrogen appeared in the precipitate until pH 7.9 was exceeded. Thus there is a considerable range of hydrogen ion concentration within which a separation of the bases may apparently be made.

A series of separations was next carried out at different hydrogen ion concentrations upon solutions containing sufficient known quantities of the two bases to permit recovery as crystalline derivatives. The data obtained are presented in Tables I and II. Several modifications in detail were introduced as the work progressed. The method, as finally adopted (Experiments 5 and 6), was as follows.

#### *Method of Separation.*

To the solution containing the bases and a small amount of sulfuric acid, used to dissolve the arginine carbonate, at a volume of approximately 500 cc., boiling saturated silver sulfate solution was added until a spot test with barium hydroxide showed the presence of excess silver. This required about 800 cc. The solution was cooled and cold saturated barium hydroxide solution was added from a graduated cylinder until, on stirring, the precipitate took on a faint buff color. At this point the solution was practically neutral to litmus paper and the precipitate flocculated

<sup>1</sup> We wish to express our thanks to Dr. Florence Fenwick, National Research Council Fellow in Chemistry at Yale University, for attempts made, at our request, to develop an electrometric method for the measurement of the hydrogen ion activity of these solutions. Owing to the presence of a noble metal the hydrogen electrode could not be used and the oxygen electrode, employed on the assumption that the silver compounds of the bases might be hydroxides, was likewise found to be useless. The development of the simple colorimetric technique rendered further experiments unnecessary.

sharply. A 5 cc. sample was withdrawn, centrifuged, the clear fluid treated with a few drops of 0.04 per cent brom-thymol blue indicator solution prepared according to Clark's directions, and the color compared with that of sealed tubes containing appropriate buffer solutions and the same indicator.<sup>2</sup> The test solution and precipitate were then returned to the main solution and addition of barium hydroxide continued until a sample, tested in this way, matched the color of the buffer at pH 7.0. It is evident from the data of Table I that some latitude is permissible in adjusting this reaction, and that our selection of pH 7.0 is somewhat arbitrary. Experiment 4, however, indicates that the acidity must be somewhat less than pH 6.6 as at this reaction some histidine escaped precipitation.

After about 15 minutes of careful stirring the precipitate was centrifuged and the clear supernatant fluid poured off and acidified to litmus with sulfuric acid. The precipitate was suspended in water to which a minimal amount of hydrochloric acid was added, and boiled to ensure complete decomposition of the histidine silver. Excess of boiling saturated silver sulfate was then added, the solution cooled and again brought to pH 7.0 by the cautious addition of barium hydroxide solution, again carefully stirred, and the precipitate of silver chloride, barium sulfate, and histidine silver centrifuged off. The supernatant fluid was removed, acidified, added to that from the first precipitation, and the whole concentrated *in vacuo* to about 800 cc.

By this double precipitation the difficult washing of the first histidine silver precipitate was avoided and the quantity of arginine in the histidine fraction materially reduced, as is clear when Experiments 3 and 4, in which a single precipitation was employed, are compared with Experiment 6 (Table I, Column 6).

The second histidine silver precipitate was suspended in water, treated with a small excess of hydrochloric acid, and boiled, the precipitate of silver chloride, etc., centrifuged off and thoroughly washed with hot water. The solution containing the histidine was then evaporated to dryness *in vacuo* to remove the excess of hydrochloric acid, made to standard volume, and nitrogen determined in an aliquot part.

<sup>2</sup> The buffer solutions prepared by the LaMotte Chemical Products Company are satisfactory.

The remaining portion of the solution was evaporated to approximately 30 cc. and histidine recovered as di-salt of dinitronaphtholsulfonic acid in two successive crops (4).

The concentration of the acidified filtrates from the histidine silver permits the precipitation of arginine silver from a relatively small volume of solution and avoids much of the uncertainty aroused by the possibility that this substance is not wholly insoluble. We have found that a little silver sulfate usually separates from this solution on concentration, and, when necessary, have removed it by filtration.

Arginine silver was precipitated by the addition of warm saturated barium hydroxide solution until the reaction was strongly alkaline to phenolphthalein, centrifuged off, suspended in water, and decomposed by warming with a small excess of dilute hydrochloric acid. Since a small amount of barium was always present, dilute sulfuric acid was added drop by drop until a sample, removed and centrifuged, showed the presence of a trace of sulfate ion. The mixture of silver chloride and barium sulfate, which also contained a trace of adsorbed indicator from the previous tests, was centrifuged off and washed, the solution concentrated to dryness *in vacuo*, and made to standard volume. An aliquot part was removed for total nitrogen determination and the arginine in the remainder recovered by precipitation with a small excess of dinitronaphtholsulfonic acid.

Saturation of the solution with powdered barium hydroxide in order to precipitate arginine silver, as recommended by Kossel, is wholly unnecessary. While we have not ascertained the exact hydrogen ion concentration at which arginine silver is completely precipitated, it probably lies near or somewhat beyond pH 10. Provided the solution was made strongly alkaline to phenolphthalein, we have encountered no case in which appreciable amounts of arginine escaped precipitation.

In order to obtain information regarding the completeness of the separation, it was necessary to analyze the histidine solution for arginine and the arginine solution for histidine. The determination of small amounts of arginine in a solution containing relatively large amounts of histidine is, however, difficult and somewhat uncertain. We have employed Van Slyke's method of decomposition with sodium hydroxide and estimation of the



ammonia thereby produced. In the earlier experiments (Nos. 1 and 2, Table I) Plimmer's (5) modification of this method was used, in which, at the end of the 6 hour digestion, the water is withdrawn from the condenser and the ammonia distilled up into the absorption apparatus at the top. This method was later abandoned, as difficulty was experienced in completing the distillation, and the convenient modification suggested by Koehler (6) was employed, in which a slow stream of air, passing through the flask, sweeps the ammonia into an absorption apparatus.

The fundamental difficulty with this estimation in the present case is, however, the instability of histidine. Plimmer (5) has published some experiments which show that 1.5 to 3 per cent of the nitrogen of histidine is split off as ammonia by a 6 hour digestion with strong sodium hydroxide solution (50 or 20 per cent). We have verified these findings using our purest preparation of free histidine. Our experiments indicate that a somewhat variable decomposition occurs with the evolution of an average proportion of approximately 2 per cent of the histidine nitrogen as ammonia. While this decomposition is probably not a serious matter in ordinary determinations of arginine, it involves a large correction in our application of the method to the determination of traces of arginine in the presence of much histidine. We therefore submit the data (Table I, Column 6) we have obtained with some reservation.

To illustrate the calculation of the arginine content, the data from Experiment 5 may be given. 25 cc. of this solution contained 0.01945 gm. of nitrogen. Duplicate determinations of the ammonia nitrogen evolved on 6 hours digestion with 30 per cent sodium hydroxide solution gave 0.0011 and 0.0010 gm. of nitrogen. 2 per cent of the total nitrogen in each aliquot is 0.0004 gm. of nitrogen. Deducting this we have 0.00065 gm. of nitrogen as ammonia, presumably derived from the arginine in 25 cc., or 0.0065 in the whole 250 cc. of solution. Twice this or 0.013 gm. is therefore the arginine nitrogen present in the solution.

In order to test for the presence of histidine in the arginine fraction, Hopkins' mercuric sulfate reagent was employed. As the presence of chlorides is inadmissible when this reagent is used, in the first four experiments the arginine silver precipitate was decomposed with hydrogen sulfide in the presence of dilute sul-

furic acid, silver sulfide removed, the solution brought to a standard volume, and an aliquot removed for nitrogen determination. Hopkins' reagent was then added in large excess to the remainder of the solution. Only in the experiment in which the separation was effected at pH 6.6 (Table I, Column 8, Experiment 4) was there evidence of more than a trace of precipitate of the histidine compound. In this case filtration and determination of nitrogen was possible, but unfortunately a part of the precipitate was lost through an accident. The nitrogen determination on the remainder indicates the presence of an appreciable quantity of histidine. In the other experiments only the slightest turbidity developed on standing 48 hours.

TABLE III.

*Data Showing Inapplicability of Correction for Solubility of Arginine Silver in Saturated Barium Hydroxide to Conditions Employed in These Experiments.*

Experiment No.	Arginine N taken.	N in arginine fraction.	Arginine in histidine fraction.	Volume of filtrate from arginine silver.	Solubility correction, 0.0116 gm. N per liter.	Total correction. (4) + (6)	Corrected arginine N found. (3) + (7)	Arginine N found corrected for arginine in histidine fraction. (3) + (4)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	gm.	gm.	gm.	cc.	gm.	gm.	gm.	gm.
1	0.357	0.343	0.014	1940	0.0225	0.036	0.379	0.357
2	0.357	0.345	0.017	1920	0.0222	0.039	0.384	0.362
3	0.469	0.435	0.019	2320	0.0269	0.046	0.481	0.454
5	0.485	0.468	0.013	1280	0.0148	0.028	0.496	0.481
6	0.447	0.447	0.007	1150	0.0133	0.020	0.467	0.454

The arginine fraction, in those experiments in which Hopkins' reagent had been used, was freed from mercury with hydrogen sulfide, and barium hydroxide added until the solution was only faintly acid to litmus. The barium sulfate was then removed, the solution concentrated to approximately 200 cc., and the arginine precipitated with a slight excess of dinitronaphtholsulfonic acid. The somewhat low recoveries of arginine in the first three experiments recorded in Table II are partly due to the unavoidable losses involved in the removal of mercury and sulfuric acid.

It has always been customary, in carrying out an analysis of the bases from proteins by Kossel's method, to apply a correction for the solubility of arginine silver in the saturated solution of barium hydroxide from which this compound is precipitated. This correction (1) is founded upon Gulewitsch's (7) determination of the solubility of arginine silver in saturated barium hydroxide which amounted to 0.036 gm. of arginine, or 0.0116 gm. of arginine nitrogen, per liter. When the precipitation of arginine silver is carried out at a strongly alkaline reaction to phenolphthalein, which is probably within the limits pH 10 to 11, and therefore not far from the isoelectric point of arginine (pH 10.97) (8), this correction is not necessary. The data in support of this statement are contained in Table III. Comparison of Columns 8 and 2 of this table shows that the corrected arginine nitrogen is in every case materially higher than the arginine nitrogen taken. When, however, the correction for the arginine nitrogen found in the histidine fraction alone is added (Column 9) the nitrogen recoveries are within the error of such experiments.

*Purification of Arginine.*—The arginine employed was a preparation of crude arginine carbonate that had been obtained from casein by the Kossel-Kutscher procedure. Analysis by the Van Slyke method indicated that it was approximately 95 per cent pure. We have found that the best salt for the further purification of arginine of this grade is the picrate which possesses convenient solubilities in water and is readily prepared by adding the theoretical amount of pure picric acid, dissolved in hot alcohol, to an aqueous solution of the carbonate. The picrate separates, on standing overnight, in large masses of yellow needles which decompose, when heated slowly, at 217–218°C. Slightly higher decomposition points could be obtained by rapid heating but this point was characteristic. Riesser (9), who also employed this method of purification, gave the decomposition point of arginine picrate as 205–206°. Our decomposition point was obtained with a short stem thermometer and was unchanged by recrystallization. The salt contains 2 molecules of water of crystallization, theory 8.20 per cent, found 8.18 per cent.

The picrate was decomposed with hot 10 per cent sulfuric acid, picric acid removed with ether, and a faint trace of yellow color removed with norit. Sulfuric acid was exactly removed by the addition of the requisite amount of pure barium hydroxide, the barium sulfate centrifuged off and thoroughly washed with boiling water. On concentration *in vacuo* to a thin sirup and scratching, free arginine slowly separated in short, thick, clear prisms. The crystals rapidly disintegrate into a white powder in the air with formation of the carbonate. Free arginine, rapidly dried between

filter papers, darkened at 223° and decomposed at 238°. This is much higher than the decomposition point given by Gulewitsch (7) of 207–207.5°. In order to make certain that our higher decomposition points were not due to racemization we determined the rotation. A 16.27 per cent solution gave  $[\alpha]_D^{25} = +12.5^\circ$  and the same solution diluted to 8.13 per cent gave  $[\alpha]_D^{25} = +12.86^\circ$ . Gulewitsch found that the specific rotation of a 9.6 per cent solution of the chloride which contained an excess of barium hydroxide was  $+12.94^\circ$ . Our product was therefore probably optically pure. The preparation was converted to the carbonate and dried over lime.

*Purification of Histidine.*—A preparation of histidine dichloride obtained by the method of Hanke and Koessler (10) from blood cells was found to retain some impurity even after several recrystallizations. Since free histidine can be crystallized from water and even better from 50 per cent alcohol, the dichloride was treated, in 10 or 20 gm. lots, with excess of hot silver sulfate solution, silver chloride removed, excess silver precipitated as sulfide and the solution concentrated slightly to remove hydrogen sulfide. It was then brought to pH 7.2 by the addition of pure barium hydroxide. Barium sulfate was then removed and washed, and the solution was evaporated *in vacuo* to crystallization. The addition of an equal volume of absolute alcohol at this stage materially increases the yield of pure substance.

The decomposition point of free histidine is somewhat unsatisfactory. One of our preparations darkened at 255° and decomposed with effervescence at 265° on slow heating. When rapidly heated it darkened at 260° and decomposed at 280°. Abderhalden and Weil (11) described preparations of free histidine which darkened from 255–260° and decomposed at 279–280° on rapid heating. We have prepared one specimen which was crystallized twice as chloride, once as di-salt of dinitronaphtholsulfonic acid, and twice as free base, which darkened from 250° and decomposed at 277° on slow heating.

The purified histidine when dissolved in water gave a solution, the hydrogen ion concentration of which lay at pH 7.2 as determined by means of brom-thymol blue or phenol red. Since the isoelectric point of this substance is pH 7.15 (12) this furnishes a sensitive test of its purity. It was noted that samples obtained from the mother liquors of the purer material gave solutions which were distinctly more alkaline. Moreover a solution of pure histidine on boiling became slightly alkaline. Free histidine is therefore somewhat unstable.

*Recovery of Dinitronaphtholsulfonates.*—The data obtained from Experiment 6 (Table II) may be given to illustrate the details of the recovery of the dinitronaphtholsulfonates of histidine and arginine.

The histidine solution contained 0.232 gm. of nitrogen in 250 cc. Of this 180 cc. were concentrated to about 30 cc. and 3.2 gm. (2.5 gm. = 2 mols) of dinitronaphtholsulfonic acid were added. On standing overnight 2.966 gm. (dried at 105°) of di-salt separated. This decomposed at 252° after darkening from 240°. On concentration the mother liquor yielded a further crop of 0.097 gm. These two crops contained histidine nitrogen equivalent to 0.228 gm. based on the whole solution.

The arginine solution contained 0.447 gm. of nitrogen in 250 cc. Of this 240 cc. were concentrated slightly and 4.2 gm. (3.8 gm. = 1 mol) of dinitronaphtholsulfonic acid added at boiling temperature. The salt promptly separated from the hot solution in orange plates with a golden yellow luster and after standing overnight 3.473 gm. (dried at 105°) were obtained, nitrogen, found 16.9 per cent, theory 17.2 per cent. The mother liquor was concentrated to 10 cc. and on standing yellow crystals separated. These were filtered off and boiled with a little water when a part dissolved and the insoluble residue became orange.<sup>3</sup> On further standing 0.099 gm. of orange plates was obtained. These two crops contained arginine nitrogen equivalent to 0.427 gm. based on the whole solution.

An investigation of the application of this method of separating histidine from arginine to the analysis of the bases derived from protein is in progress. Preliminary experiments have already shown that the method greatly facilitates this analysis and also indicates that it may be applied to the preparation of arginine and histidine on a large scale. Investigation of this is also planned. In this connection it may be well to draw attention to the recent suggestion of Kiesel (13) who avoids the large volumes required when much silver sulfate must be added to a solution by adding the silver in the form of a paste of the oxide, maintaining the acidity of the solution by the addition of sulfuric acid as needed.

The fact that both histidine and arginine are completely precipitated as silver compounds in the vicinity of their respective isoelectric points, is very suggestive. While we are ignorant of the exact nature of these compounds it would seem that the reaction takes place between silver ion and the organic base in its undissociated form. This point deserves detailed study.

The general principle underlying this method of separation can probably be quite widely applied. We have already employed precipitation of silver compounds at acid, neutral, and alkaline reactions as a convenient and rapid means of preparing purine, histidine, arginine and lysine fractions from yeast extracts. Much investigation of the details of the procedure is, however, necessary in this more complicated case.

<sup>3</sup> We suspect that arginine may, like histidine, form two compounds with dinitronaphtholsulfonic acid. This behavior will be investigated.

## SUMMARY.

Histidine can be approximately completely separated from arginine by bringing the reaction of a solution of the two bases, which also contains an excess of a soluble silver salt, to pH 7.0. By a second precipitation as silver compound a histidine fraction is obtained which contains no material quantity of arginine.

No appreciable amount of histidine escapes this precipitation, the arginine fraction being practically free from this base.

Arginine silver is subsequently precipitated at a strong alkaline reaction to phenolphthalein (pH 10 to 11). Saturation of the solution with barium hydroxide in order to precipitate arginine silver is shown to be not only unnecessary but inadvisable.

The silver precipitates can be conveniently decomposed with hydrochloric acid and the bases recovered very completely as salts of dinitronaphtholsulfonic acid.

The method offers many advantages over that previously employed for the separation of these bases as the entire separation can be performed in a few hours. The application to the analysis of the bases from proteins and to the preparation of arginine and histidine in large quantities will be developed.

It is suggested that the general principle of silver precipitations at different hydrogen ion concentrations may receive a much wider application.

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## THE SPECIFIC EFFECT OF SALTS IN THE EXTRACTION OF UREASE FROM THE AMEBOCYTES OF LIMULUS.

BY LEO LOEB AND OSCAR BODANSKY.

(From the Department of Pathology, Washington University, School of Medicine, St. Louis, and the Marine Biological Laboratory, Woods Hole.)

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In a former communication we have shown urease to be present in the amebocyte tissue, in the blood plasma, muscle tissue, and even in the non-fertilized eggs of *Limulus*; on the other hand we did not find urease in tissues of lobster and spider-crab.<sup>1</sup> Preliminary experiments indicated that substances differ in their ability to extract the urease from the tissues. During the past season we undertook a more complete series of experiments in which we compared the power of various substances, especially of salts, to extract urease. In the course of our investigations we discovered very marked differences in the extracting power of certain salts.

*Method of Testing the Strength of the Extracts.*—Our method of testing the extracts was as follows: We added to 25 cc. of 1 per cent urea solution 25 cc. of extract. The samples were kept in an incubator at a temperature of 30°C.; fluctuations from this temperature did not exceed 1°. In each experiment, in which we compared the effectiveness of various extracts, the latter acted on the urea solution during the same period of time; in many cases the length of time was 24 hours, more or less, but in all cases the period was always the same for the various extracts in the same experiment. The ammonium carbonate formed was determined by Van Slyke's method; however, it was found necessary to continue the aeration for a period lasting from 2 to 3 hours even when the solution was saturated with sodium carbonate. The ammonium was driven over into M/25 HCl and the excess acid titrated with M/25 NaOH, sodium alizarin sulfonate serving as indicator. The

<sup>1</sup> Loeb, L., and Bodansky, O., *J. Biol. Chem.*, 1926, lxxvii, 79.



values given in the various tables are the number of cc. of  $M/25$  HCl neutralized by the ammonia.

Instead of comparing enzyme concentrations by determining the reciprocals of the times necessary to effect a certain definite change in the substrate, we used for this purpose the amounts of ammonium carbonate produced in a definite period of time. Since proportionality between amount of enzyme and change effected exists only when the latter is a small percentage of the total substrate, this method of comparison introduces an error of such a nature that the values found for extracts which cause large changes will be relatively too low. In reality, therefore, the differences which we observed between more effective and less effective extracts represent minimum values, the efficiency of the more effective extracts being generally greater than indicated in our figures. This method of comparing enzyme concentrations in different experiments is therefore only roughly quantitative, but in view of the range of fluctuations, which are due to the method of extraction, it was considered sufficient for our purposes.

*Effect of pH in Urease Action.*—For the same reason it was not considered necessary to maintain a constant pH in the mixture of extract and urea solution. The following tests show that the presence of buffer did not influence the values of urease action sufficiently to make its use of special value in our experiments. It would thus seem that under our conditions of experimentation the possible increase in velocity of reaction produced by using phosphate buffer to eliminate the excess of OH ions due to the formation of ammonium carbonate is more than counteracted by the retarding effect of the phosphates, considering the degree of accuracy in our experiments.

*Effect of Buffer on NaCl Extracts.*—In a mixture of 25 cc. of a NaCl extract of amebocyte tissue with 25 cc. of urea solution enough ammonia was produced to neutralize 7.4 cc. of  $M/25$  HCl (final pH 9.1). On the other hand, 25 cc. of the same extract added to 25 cc. of urea solution buffered with 0.5  $M$  phosphate of pH 6.6 gave a titration value of 2.3 (final pH 6.6). The results are shown in Table I.

Another experiment checked this result.

Tissue 66. NaCl extract + urea, titration value 5.0; final pH 9.0.

“ “ + “ + 0.5  $M$  buffer value, 2.2; final  
pH 6.6.

*Preparation of Extracts.*—By means of filter paper the tissues from several *Limuli* were freed as well as possible from their serum, then thoroughly mixed in a mortar, and this mixture was divided in each experiment into quantities equal by weight for the purpose of extraction by the various substances. The weight of each of these samples was 3 gm. in the first experiments, 2.5 gm. in the later experiments, and 1.5 gm. toward the end of the season. In the various tables the quantities of tissue used for extraction are recorded. The tissue was then ground in the mortar with a weighed amount of sand (1.7 gm. of sand for 2.5 gm. of tissue and 1.0 gm. of sand for 1.5 gm. of tissue) for a period totalling 1.5 minutes in each case; this was followed by the addition of 40 cc. of the extracting solution to the ground material. Both were

TABLE I.

*Effect of Phosphate Buffer of pH 6.6 upon the Action of 0.5 Gm. of Amebocyte Tissue on 25 Cc. of 1 Per Cent Urea.*

Tissue 66 used. Time 24 hrs.

Experiment.	Buffer.	Final pH.	Titration with HCl.
	<i>M</i>		<i>cc.</i>
A	None.	9.1	52.9
B	0.05	7.8	37.4
C	0.10	7.2	39.2
D	0.20	6.7	30.2
E	0.40	6.6	38.5
F	0.50	6.6	45.0

mixed by stirring and allowed to remain in contact under toluene for about 1 to 2 hours, the length of time being the same for all samples in the same experiment. The fluid was then filtered off from the mixtures and the enzyme concentration of the filtrates was determined by adding 25 cc. of the extract to 25 cc. of a 1 per cent urea solution, allowing the action to take place during a definite period of time, which is mentioned in each experiment. The salts contained in the extract were not removed through dialysis. In case the amount of salt present in the extract was relatively great, it may possibly have exerted a retarding effect on the enzyme action. This factor will have to be considered in evaluating our results. The amount of ammonium carbonate produced

TABLE II.

*Extractions with Distilled H<sub>2</sub>O, Isotonic NaCl, Sea Water, Serum Filtrate, and Heated Serum.*

T designates No. of tissue used in extraction; t the number of hrs. of action of extract on urea solution. The values given are number of cc. of M/25 HCl neutralized by ammonia.

Extractive.	A	B	C*	D*	E	F
	T 9, 10 t = 24	T 12, 13 t = 39.5	T 15, 16, 17, 18, 19, 20 t = 24	T 15, 16, 17, 18, 19, 20 t = 24	T 27, 28, 29, 30 t = 24	T 70, 71, 72 t = 34.5
Distilled H <sub>2</sub> O.....	0.0	0.1	0.0	0.0	1.8	
N/2 NaCl.....	2.5	7.9	1.2	18.8	7.4	4.6
Sea H <sub>2</sub> O.....	1.1					
“ “ (neutralized).....		1.2	5.6	1.0	10.6	3.6
Serum filtrate.....	3.7	6.8	2.8	12.8	3.4	
Heated serum.....	4.0	9.2	3.4	9.9	9.9	

In Experiment A 3 gm. of tissue were used for each extraction; in the others, 2.5 gm.

\* In Experiments C and D there is a considerable variation in efficiency of the extracts, although the same tissues were used in both experiments. However, Experiment D was carried out after these tissues had been kept in the ice chest 2 days longer. The variation in the results in Experiments C and D may be largely due to the fact that these experiments were among our first at a time when our method of extraction was still more variable than later.

TABLE III.

*Extraction with Urea.*

4.0 gm. of tissue were extracted with 65 cc. of 1 or 5 per cent urea solution. A 25 cc. sample was taken immediately after the extraction in order to determine the extent of ammonia formation during the time of extraction; this was subtracted from the value obtained by the action of another 25 cc. sample on 25 cc. of 1 per cent urea for 24 hrs.

Extractive.	A	B	C
	T 15, 16, 17, 18, 19, 20 t = 24	T 25, 26 t = 24	T 27, 28, 29, 30 t = 24
Distilled H <sub>2</sub> O.....	0.0	0.8	1.8
1 per cent urea.....	0.3	1.1	1.1
5 “ “ “.....		0.0	1.7

was determined by Van Slyke's method in samples of 25 cc. each. Multiplied by 2, this figure represented the amount of am-

monium carbonate formed in the total volume of 50 cc. of the mixture and this figure was used to designate the efficiency of extraction. In our first experiments our method of preparation of the extracts had not yet the same degree of exactness as in the subsequent experiments. There is therefore a greater variability of the results obtained; but notwithstanding this shortcoming the differences between extraction by  $\text{CaCl}_2$  solution and by other salts are quite evident.

In the experiments of Table II we used the ordinary Woods Hole sea water, which is quite definitely alkaline, as well as pre-

TABLE IV.  
*Comparative Extractions by Salts of Alkali Metals and by  $\text{CaCl}_2$ .*

Extractive.	A T 32, 33 t = 39	B T 34, 35, 36, 37 t = 24	C T 40, 41 t = 19
Distilled $\text{H}_2\text{O}$ .....	0.0	0.2	0.0
N/2 NaCl .....	0.5	0.7	2.9
N/2 KCl .....	0.7		
Sea $\text{H}_2\text{O}$ (neutralized) .....		1.7	1.7
Heated serum .....		8.3	4.1
Isotonic $\text{Na}_2\text{SO}_4$ .....	0.3		
“ $\text{K}_2\text{SO}_4$ .....	0.1		
$\text{CaCl}_2$ (0.38 M) .....	14.2	30.1	79.0
“ (0.38 “) .....		11.0	74.6
“ (0.38 “) .....			70.6

viously neutralized sea water. The extractive efficiency of sea water was compared with that of distilled water, sodium chloride, *Limulus* serum in which urease has been destroyed through previous heating to  $80^\circ$  for 30 minutes, and serum filtrate. *Limulus* serum was freed from its protein content by heating under pressure in the autoclave and subsequent filtration and represented, therefore, a watery solution of blood salts. We may conclude from the figures of Table II that distilled water extracts only a trace of enzyme, while N/2 NaCl, sea water, serum filtrate, and heated serum have a coefficient of approximately 6.

*Does Addition of Urea to Distilled Water Increase the Efficiency of the Latter as an Extractive?*—We determined whether a solution of urea, on which the enzyme acts, could possibly have a specific

effect on the extraction of urease from the amebocyte tissue. The experiments of Table III lead to the conclusion that this is not the case. Watery solutions of 1 and 5 per cent urea behave approximately like water, in both cases the extractive power being approximately 0.8 to 0.9.

*Comparison between the Extracting Power of Salts of Alkali Metals and  $\text{CaCl}_2$ .*—Table IV shows the comparative efficiency as

TABLE V.  
*Addition of  $\text{CaCl}_2$  to the Extract.*

Material.	t	Value without addition of $\text{CaCl}_2$ .	Value with addition of $\text{CaCl}_2$ .
S 45	25	4.5	1.3
" 46	25	5.0	5.0
" 47	25	3.5	3.5
T 46	25	43.6	64.6
" 47	25	49.5	49.7
NaCl Ex. T 66	25	5.0	2.2

S = *Limulus* serum; T = amebocyte tissue; Ex. = extract of amebocyte tissue.

TABLE VI.  
*Addition of  $\text{CaCl}_2$  to  $\text{H}_2\text{O}$  Extract.*

Extractive.	A	B
Distilled $\text{H}_2\text{O}$ .....	0.7	
" " acting as extract for 15 min.;		
$\text{CaCl}_2$ is added, then urea.....	0.6	1.4
$\text{CaCl}_2$ (0.38 M).....	155.2	165.0

extractives of  $\text{H}_2\text{O}$ , alkali metals, and  $\text{CaCl}_2$ . Water is again very ineffective, its coefficient being below 1. NaCl, KCl approximately isotonic with sea water, sea water, isotonic  $\text{Na}_2\text{SO}_4$  and  $\text{K}_2\text{SO}_4$ , and heated *Limulus* serum have a coefficient ranging between 1 and 6. Isotonic  $\text{CaCl}_2$  is about 20 to 40 times as effective as isotonic NaCl. We notice that the efficiency of the extracts obtained from different tissues varies, but there is a certain parallelism between the relative efficiency of various extracting substances in the case of the different tissues.

*Neutralization of Ammonium Carbonate by  $\text{CaCl}_2$  Extracts.*—

The NaCl solution used for extraction had a pH of about 7.0. Directly after extraction the NaCl extract had the same pH. After action upon urea solution, the pH rose to about 9.0. This rise was observed in almost every experiment and was due to the formation of ammonium carbonate. In the case of the  $\text{CaCl}_2$  extract, however, the pH, originally 7.0, rose, after action on the urea solution, only to about 7.2. However, the great activity of the  $\text{CaCl}_2$  extracts and the large amounts of ammonium carbonate formed in the urea solution by these extracts cannot be due to the maintenance of this favorable pH throughout the action, as is

TABLE VII.  
*Extractions by Salts (Chlorides) of Various Metals.*

Extractive.		A T 58, 59, 60, 61 t = 38.5	B T 70, 71, 72 t = 34.5	C t = 18	D t = 19.5	E t = 14.5
Alkali metals.	NaCl.....	3.6	4.6	1.2	2.4	2.0
	KCl.....	5.0				
		4.6				
Alkaline earths.	$\text{CaCl}_2$ .....	197.6	140.6	110.0		
	$\text{BaCl}_2$ .....	192.0	71.8	115.4		
	$\text{SrCl}_2$ .....	128.4	162.4			
	$\text{MgCl}_2$ .....	11.0	37.6	41.4		43.0
	$\text{MnCl}_2$ .....		43.8	44.0		7.0
	$\text{ZnCl}_2$ .....				0.8	0.4
	$\text{CuCl}_2$ .....				0.2	

In these experiments we used 1.5 gm. of tissue ground with 1 gm. of sand and extracted with 40 cc. of solution.

shown by our experiments in which phosphate buffer was added to the urea solution and extract or amebocyte tissue mixtures, and a result comparable to that observed in the case of the  $\text{CaCl}_2$  extracts was not obtained (Table I).

*Does Addition of  $\text{CaCl}_2$  to the Completed Extracts Exert an Effect on Their Potency?*—After having established the very marked potency of  $\text{CaCl}_2$  extracts of amebocyte tissue on urea solution, we considered the possibility that the presence of  $\text{CaCl}_2$  in the mixture of urea solution and amebocyte tissue or NaCl extracts of amebocyte tissue might be entirely or partly responsible for this effect. However, in experiments in which we added a quantity of

$\text{CaCl}_2$  equivalent to that present in our mixtures of urea solutions and  $\text{CaCl}_2$  extracts of amebocyte tissue to mixtures of urea solutions with heated *Limulus* serum or amebocyte tissue or  $\text{m}/2$   $\text{NaCl}$  extract of amebocyte tissue, such addition of  $\text{CaCl}_2$  did not have a significant effect on the splitting of urea. In Table V we compare the effect on urea solution of heated *Limulus* serum, amebocyte tissue, and  $\text{m}/2$   $\text{NaCl}$  extract of amebocyte tissue with and without addition of  $\text{CaCl}_2$ .

This conclusion is confirmed by the experiments of Table VI. We compared the effectiveness of the ordinary water extracts of

TABLE VIII.

*pH Changes during Extraction and Action of Extract on Urea.*

All extractions were brought to pH 7.0 by addition of a small amount of acid or alkali before they were added to the amebocyte tissue.

Solution.	A T 58, 59, 60, 61 t = 38.5		B T 70, 71, 72 t = 34.5		C Mixtures of T t = 18		D Mixtures of T t = 14.5	
	pH of extract.	pH after urease action.	pH of extract.	pH after urease action.	pH of extract.	pH after urease action.	pH of extract.	pH after urease action.
$\text{NaCl}$ .....	6.7	8.7		9.1				7.8
$\text{KCl}$ .....	6.7	9.0						
$\text{CaCl}_2$ .....	6.6	7.2		6.9		7.3		
$\text{BaCl}_2$ .....	6.6	6.7		7.4		7.0		
$\text{SrCl}_2$ .....	6.6	7.3		6.9				
$\text{MgCl}_2$ .....	5.0	8.5		9.1		9.1		9.1
$\text{MnCl}_2$ .....				6.6		6.4		6.7
$\text{ZnCl}_2$ .....								

amebocyte tissue with that of extracts which had been prepared by extracting amebocyte tissue in the ordinary way by water with the addition of sand, except that in this case the water acted only during a period of 15 minutes on the amebocyte tissue, and that after this extraction at once an amount of  $\text{CaCl}_2$  was added to the filtered extract which made the concentration of  $\text{CaCl}_2$  equal to the concentration present when a  $0.38 \text{ M}$   $\text{CaCl}_2$  solution is used for extraction. The extract with the added  $\text{CaCl}_2$  was then mixed with the urea solution. At the same time a  $0.38 \text{ M}$   $\text{CaCl}_2$  solution of amebocyte tissue was prepared in the usual way and mixed with urea solution. It was found that subsequent addition of  $\text{CaCl}_2$

to the water extract did not increase in a significant way the effectiveness of the water extract, and that the  $\text{CaCl}_2$  extract was approximately 150 times as efficient as the water extract with or without subsequent addition of  $\text{CaCl}_2$ . It is not very probable, therefore, that  $\text{CaCl}_2$  acts by protecting the urease, after it has been extracted, from destruction.

*Comparison of the Extracting Power of Salts with Different Kinds of Cations, and of the Neutralizing Effect of Extracts Thus Prepared.*—In this experiment we compared extracts made with isotonic solutions of salts of alkali metals, alkaline earths, and heavy metals (Table VII). Chlorides were used; thus the anion was the same in all cases.

Again the effectiveness of  $\text{NaCl}$  and  $\text{KCl}$  were of a similar order. The coefficients of extractive power of these salts vary between 1 and 5, depending upon the kind of tissue used and certain accidental factors.  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , and  $\text{BaCl}_2$  were all of similar effectiveness; their coefficient ranged between 72 and 197, being on the average 140. Therefore isotonic solutions of the chlorides of the heavy metals were about 30 to 40 times more effective than isotonic solutions of the alkali chlorides. The extracting coefficient of  $\text{MgCl}_2$  stood between that of the chlorides of  $\text{Ca}$ ,  $\text{Ba}$ , and  $\text{Sr}$  on the one hand, and that of the alkali chlorides on the other hand; its average was about 30; it was, however, nearer that of the latter than of the former.  $\text{MnCl}_2$  behaved similarly to  $\text{MgCl}_2$ ; however, there was in one experiment a somewhat lower figure obtained (7.0), although even in this case it was higher than the corresponding figure for alkali salts. A somewhat similar range was also found in the case of  $\text{MgCl}_2$ . The extracts made with salts of heavy metals ( $\text{Zn}$ ,  $\text{Cu}$ ) showed very little activity; their coefficient was below 1. Since the metals were not removed from the extract before the latter was added to the urea solution, it is possible that the low degree of activity is due to an injurious effect of these substances on the enzyme as well as to their failure to extract the enzyme (Table VIII).

We have stated that in the mixture of  $\text{CaCl}_2$  extract of amebocyte tissue and urea solution, the pH remains approximately neutral during the action of the urease;  $\text{BaCl}_2$  and  $\text{SrCl}_2$ , which are equally effective, act in this respect in the same way.  $\text{MgCl}_2$  extract, on the other hand, behaves like the alkali salt extracts;



the pH in the mixture rises to approximately 9.0 as the result of the ammonium carbonate formation; in one case in which the NaCl extract acted for 14½ hours on the urea solution the pH rose only 7.8.  $\text{MnCl}_2$ , which was about as active as  $\text{MgCl}_2$ , differed from the latter by keeping the pH slightly on the acid side. As we have previously stated, it is not very probable that the effectiveness of the Ca, Ba, and Sr salts depends upon this regulation of the pH; there is no parallelism between the regulation of the pH by various extracts and the extractive power of various salts; and, as shown, through the addition of buffers to other extractives it is not possible to imitate the effect of  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ , or  $\text{SrCl}_2$ .  $\text{MgCl}_2$  and  $\text{MnCl}_2$  are about equally effective; yet the end-pH in both cases differs greatly.

*Cause of the Neutralizing Power of Extracts Prepared with  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ , and  $\text{SrCl}_2$  Solutions.*—We carried out some experiments in which we tried to determine whether the presence of the  $\text{CaCl}_2$  in the mixture of extract and urea solution is responsible for the lower pH in case the  $\text{CaCl}_2$  extracts acted on amebocyte tissue. The  $\text{CaCl}_2$  extract behaved in about the same manner as in the previous experiments. It was very active. The pH in the mixture was only very little raised on the alkaline side. In one experiment in which the calcium in the extract was precipitated through addition of oxalate solution before the extract was mixed with the urea solution, the effectiveness of the extract was much diminished, but it was still much higher than in the NaCl extracts; the pH now behaves as in NaCl extracts. It is probable that the formation of Ca oxalate caused the elimination of a large quantity of the urease. However, it will be necessary to repeat this experiment before we can consider this conclusion as definite. As we stated previously, addition of  $\text{CaCl}_2$  to the NaCl extract does not increase the efficiency of this extract, and the pH remains higher than in the active  $\text{CaCl}_2$  extract; neither does the addition of phosphate buffer to NaCl extract increase the enzymatic potency of this extract (see Table IX).

6.0 gm. of a mixture of tissues were ground with a corresponding amount of sand and extracted with 96 cc. of isotonic calcium chloride solution. 7.5 gm. of the same mixture of tissue were ground with a corresponding amount of sand and extracted with 120 cc. of isotonic NaCl solution.

There remained still the possibility that some substance is extracted by  $\text{CaCl}_2$ , which is not extracted, or is extracted in a very much smaller quantity by  $\text{NaCl}$ , which has the power to neutralize alkali. We compared, therefore, the titration curves in 25 cc. samples of  $\text{CaCl}_2$  and  $\text{NaCl}$  extracts in order to determine the buffer action of these two extracts. The pH was tested by removing a drop from the extracts after addition of measured quantities of  $\text{N}/100$   $\text{NaOH}$  and testing colorimetrically. The

TABLE IX.  
*Effect of Oxalate on  $\text{CaCl}_2$  Extract.*

Time, 24 to 25 hrs. in all cases.

		Cc. of M/25 HCl.	pH after urease action.
A	25 cc. $\text{CaCl}_2$ extract + 25 cc. urea . . . . .	113.2	7.5
B	25 cc. $\text{CaCl}_2$ extract + equivalent amount of sodium oxalate; filtered and volume measured; 25 cc. of filtrate + 25 cc. of urea; result corrected for total volume of filtrate . . . . .	21.8	9.1
C	25 cc. $\text{NaCl}$ extract (Tissue 66) + 25 cc. urea.	5.0	9.0
D	25 cc. $\text{NaCl}$ extract + $\text{CaCl}_2$ of such concentration that it would be 0.19 M in total volume of 50 cc.; urea solution of such concentration that the urea concentration would be $\frac{1}{2}$ per cent in total volume . . . . .	2.2	8.4
E	25 cc. $\text{NaCl}$ extract + 0.5 M phosphate buffer to give pH of 6.6 . . . . .	2.2	6.6

figures in Table X show that the buffer action towards alkali is decidedly greater in  $\text{CaCl}_2$  extract than in the  $\text{NaCl}$  extract. This experiment also will have to be repeated before we can consider this result as definite.

There is then, as far as we can judge at the present time, some substance present in the  $\text{CaCl}_2$  extract of amebocyte tissue which has the power to neutralize  $\text{NaOH}$  solution more efficiently than the substances found in  $\text{NaCl}$  extract.

It was conceivable that the greater neutralizing power of  $\text{CaCl}_2$  extracts as compared with that of  $\text{NaCl}$  extracts was due to a greater content in proteins in the former which functioned as buffers towards alkali. We tried therefore to compare the protein content of  $\text{CaCl}_2$  and  $\text{NaCl}$  extracts by means of Kjeldahl determination.

A 12.5 cc. sample of a  $\text{CaCl}_2$  extract, prepared in the usual way, by mixing 1.5 gm. of tissue and 40 cc. of fluid, gave, on Kjeldahl

TABLE X.  
*Neutralizing Power of NaCl and CaCl<sub>2</sub> Extracts.*

CaCl <sub>2</sub> extract.		NaCl extract.	
N/100 NaOH added.	pH	N/100 NaOH added.	pH
0.00	6.4	0.00	6.4
0.43	6.4	0.15	6.8
0.85	6.8	0.50	6.9
1.35	7.0	1.10	7.6
2.10	7.1	1.60	7.8
2.95	7.8	2.05	8.2
3.20	7.9	2.65	8.5
3.90	8.2	3.40	8.9
4.40	8.4	3.85	9.1
4.75	8.5	4.25	9.3
5.35	8.7		
6.10	8.8		
7.10	8.9		
7.50	9.0		
7.90	9.2		
8.40	9.3		
9.10	9.3		

determination, a value of 0.0082 gm. of N, or using factor 6.25, 0.051 gm. of protein. A 12.5 cc. sample of a  $\text{NaCl}$  extract of the same tissue gave a value of 0.0067 gm. of N or 0.042 gm. of protein. In a second experiment 2 gm. samples of amebocyte tissue were extracted each with 50 cc. of isotonic  $\text{CaCl}_2$  and isotonic  $\text{NaCl}$  solutions; 25 cc. samples were taken for Kjeldahl tests.

$\text{CaCl}_2$  extract: 0.0182 gm. N or 0.114 gm. protein.

$\text{NaCl}$  " : 0.0147 " " " 0.094 " "

Thus while the enzyme activity of  $\text{CaCl}_2$  extracts is usually as much as 40 to 100 times and at the least is 20 times as great as that of  $\text{NaCl}$  extracts (Tables IV and VII), the amount of protein extracted is only 1.2 times as great. Both the  $\text{NaCl}$  and the  $\text{CaCl}_2$  extracts give Biuret and Millon's tests for protein. Blank experiments were also made to determine whether  $\text{NaCl}$  or  $\text{CaCl}_2$  solutions extracted any ammonium compounds from the tissue. It was found that this was not the case.

TABLE XI.  
*Effect of Varying Concentration of Various Extracting Solutions.*

Extractive.	A Mixture T t = 18		B Mixture T t = 18		C Mixture T t = 14.5		Remarks.
	pH after urease action.		pH after urease action.		pH after urease action.		
0.2 M $\text{NaCl}$ .		cc.	6.8	0.6		cc.	1.5 gm. tissue ground with 1 gm. sand.
0.5 " "		1.2	8.1	2.4	7.8	2.0	
1.25 " "			9.1	14.0	9.2	7.2	
2.5 " "					9.2	15.0	
0.1 " $\text{CaCl}_2$	6.8	1.0	7.2	1.4			
0.25 " "			8.3	22.6			
0.38 " "	7.3	110.0	8.0	51.0			
0.75 " "			7.0	118.2			
1.25 " "			7.0	74.2			
2.00 " "	8.2	8.2	8.2	8.0			
0.38 " $\text{MgCl}_2$	9.0	41.4			9.1	43.0	
1.25 " "					6.6	1.0	

The difference in neutralizing power of the  $\text{CaCl}_2$  extracts can therefore not be due to the greater content in protein as compared to the  $\text{NaCl}$  extract. There remains the possibility that it is due to the ability of the  $\text{CaCl}_2$  solution to extract a specific substance, perhaps of protein nature, from the amebocyte tissue which has the power to neutralize the ammonium carbonate produced in the urea solution.

*Optimal Concentration of Various Salts.*—So far we have compared only approximately isotonic solutions of the various salts. It was of interest to determine whether, through variation in the

concentration of the various salts tested, it is possible to influence their power to extract urease from the amebocyte tissue. Table XI shows the results which we have obtained so far.

The optimum concentration of the NaCl solution for extracting purposes is apparently between 1.25 and 2.5 M. The greater the urease content of the extract the higher seems to be the pH in the mixture of extract and urea solution. In the case of the CaCl<sub>2</sub> solution the optimum concentration is apparently between 0.38 and 0.75 M, at least half as low as in the case of the NaCl solution, while in the case of the MgCl<sub>2</sub> solution the optimum concentration seems to be similar to that of the CaCl<sub>2</sub> solution; but further determinations are needed in this respect. There probably enters

TABLE XII.

*Effect of Varying the Negative Ion in Extractives of the Alkali Group (Isotonic Solutions).*

	A T 32, 33 t = 39	B Mixture T t = 18		C Mixture T t = 14.5	
		pH after urease action.		pH after urease action.	
	cc.		cc.		cc.
NaCl .....	0.5	8.1	2.4	7.8	2.0
Na <sub>2</sub> SO <sub>4</sub> .....	0.3	9.2	12.8	9.1	7.6
Na <sub>2</sub> C <sub>4</sub> H <sub>4</sub> O <sub>6</sub> .....		8.8	2.8	8.3	1.9
KCl .....	0.7				
K <sub>2</sub> SO <sub>4</sub> .....	0.1				

into these results in addition to the extracting power of these salt solutions another factor; namely, the injurious effect on the enzyme of various ions or salts which differs in the case of different groups of salts. This factor increases in importance with increasing concentration of the salts used. In further experiments it will be necessary to separate these two factors and only then will it be possible to determine in an exact manner the effect of concentration on the extracting power of various salts and whether a relation exists between the valency of the cation and the optimal concentration of the salt for purposes of extraction of urease.

If we compare the amount of urease extracted by the optimal concentration of NaCl, CaCl<sub>2</sub>, and MgCl<sub>2</sub>, instead of comparing the

amounts of urea extracted by concentrations approximately isotonic with sea water, we find that  $\text{CaCl}_2$  extracts contain about 6 to 8 times as much urease as  $\text{NaCl}$  and not quite 3 times as much as  $\text{MgCl}_2$  extracts. While the difference between the urease content of the extracts of the different salts is not so great as in the case of isotonic solutions of the various salts, still it is quite striking.

TABLE XIII.

*Effect of Acid and Alkali upon Extraction of Urease by NaCl.*

After extraction the pH was tested in some cases (recorded below) and then changed to 7.0 just before addition of urea.

Extractive.	A T 43, 44 t = 24		B T 48, 49, 50 t = 24		C T 55, 56 t = 41	D T 73, 74, 75 t = 20.5	Remarks.
	pH of ex- tract.		pH of ex- tract.				
N/2 $\text{NaCl}$ .....		cc.		cc.	21.8	6.6	In A, B, C 2.5 gm. tissue ground with 1.7 gm. sand. D 1.5 gm. tissue ground with 1 gm. sand.
N/2 " N/1000 $\text{NaOH}$ .....					9.4	7.4	
N/2 " N/500 " .....			8.4	30.2	24.4	11.2	
N/2 " N/100 " .....			9.3	13.2	93.8	9.2	
N/2 " N/80 " .....		20.5					
N/2 " N/50 " .....			10.0	2.4			
N/2 " N/20 " .....			13.0	1.0			
N/2 " N/2000 $\text{HCl}$ .....						25.2	
N/2 " N/1000 " .....	6.4	29.2				28.6	
N/2 " N/500 " .....						9.0	
0.38 M $\text{CaCl}_2$ .....		155.2			165.0		

*Effects of Anions.*—We carried out a few experiments in which we tested the effect of anions in the extraction of urease. The results are shown on Table XII.

$\text{Na}_2\text{SO}_4$  seems to be somewhat more active than  $\text{NaCl}$ , but on the whole it approaches the range of activity of the latter; the same seems to hold good in the case of  $\text{K}_2\text{SO}_4$  and sodium citrate. However, it will be necessary to extend these experiments in the future.

*Effect of Addition of Acid and Alkali upon Extraction of Urease by NaCl.*—After we had established the significance of various ions in the extraction of urease, it was of interest to determine how far changes in the alkali or acid content influenced this process.

In appraising the figures of Table XIII we must consider the possibility that in addition to influencing the extraction of urease, acid and alkali may exert a direct effect on this enzyme. With this reservation, the data show that with increasing addition of NaOH the activity of the extract increases until an optimum is reached approximately between  $N/500$  and  $N/100$  NaOH. A still stronger concentration of alkali leads again to a decrease in activity. The concentration of urease extracted with the optimal alkalinity may be 2 to 4 times as strong as that with neutral NaCl. The apparent high value for extraction with NaCl in Experiment C was due, in part at least, to the fact that in this case the extract acted on the urea solution for a period of 41 hours.

To judge from a few preliminary experiments on the effect of addition of acid to NaCl solution in the extraction of urease, the optimum is at a concentration of approximately  $N/1000$  to  $N/2000$  HCl. A stronger concentration weakens again the efficiency of the extracting medium.

#### SUMMARY.

1. Different salts have a quantitatively graded effect in the extraction of urease from amebocyte tissue. If we compare solutions approximately isotonic with sea water, we find those of alkali chlorides only slightly effective, while those of Ca, Ba, and Sr chlorides are most effective; the extract prepared by means of the latter is at least 20 to 100 times as active as the former.  $MgCl_2$  and  $MnCl_2$  have an intermediate position, but as far as their activity is concerned are nearer the alkali salts. Salts of heavy metals other than  $MnCl_2$  which have been so far tested are inactive or almost inactive. Sea water, *Limulus* serum, and *Limulus* serum which has been freed from its protein content show a similar degree of activity to alkali salts or they are perhaps slightly more active. Distilled water is almost incapable of extracting the urease; addition of urea does not seem to improve the extracting power of water. It is probable that the effects observed are partly due to an injurious action of some of these substances upon the urease during the process of extraction.

2. Addition of calcium chloride to the NaCl extract immediately after the completion of the extraction does not noticeably increase the activity of the extract. This salt acts, therefore, during the process of extraction itself.

3. Various salts have an optimal concentration for the extraction of urease; this concentration is greater in the case of the monovalent cations (Na) than in the case of the bivalent alkaline earths and Mn. In this connection we must consider the possibility that relatively strong concentrations of various salts may affect the enzyme and the enzymatic process in addition to their effect on the extraction. If we compare the potency of extracts prepared with the optimal concentration of the various cations, we still find a considerably greater effectiveness of Ca, Ba, and Sr chloride than of the other salts so far tested.

4. Amebocyte extracts prepared with Ca, Sr, or Ba chloride have the power to neutralize the ammonium carbonate formed from the urea and to keep the pH at approximately 7.0. It is therefore not probable that the urease as such is the neutralizing agent. But it is possible that a substance associated with the enzyme and specifically extracted by  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ , or  $\text{SrCl}_2$  is responsible for this neutralizing power. However, the great effectiveness of  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ , and  $\text{SrCl}_2$  in extracting urease from amebocyte tissue does not depend upon the neutralizing power of these extracts.

5. Anions tested so far do not seem to possess a specific extracting power for urease from amebocyte tissue.

6. Through variation in the acid or alkali content of NaCl solutions the extracting power can be increased, but even with the optimal conditions the enzymatic activity of such extracts is considerably less than that of  $\text{CaCl}_2$  solutions.





## SULFUR IN PROTEINS.

### III. DERIVATIVES OF *l*- AND *i*-CYSTINE.\*

By ROSS AIKEN GORTNER AND WALTER F. HOFFMAN.

(From the Division of Agricultural Biochemistry, University of Minnesota,  
St. Paul.)

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In an earlier paper<sup>1</sup> we reported the action of prolonged boiling with 20 per cent HCl upon *l*-cystine. It was shown that *l*-cystine is not decomposed by such a process, but it is changed into an inactive (probably isomeric) form which we have called isomeric cystine.

This *i*-cystine has the same chemical composition as does the natural *l*-cystine but differs from *l*-cystine in many of its properties, some of which are noted as follows: (1) The *i*-cystine crystallizes in white, microscopic, needle-like crystals, while the *l*-cystine crystallizes in typical hexagonal plates. (2) The *i*-cystine is more soluble in water than is *l*-cystine. (3) The *i*-cystine is completely optically inactive. (4) The *i*-cystine reacts with the same chemical reagents as does the *l*-cystine, but the resulting derivatives differ in physical properties and crystal form from the corresponding *l*-cystine derivatives.

The two forms of cystine are similar in that both give the reducing sulfur test and are soluble in acid and in alkali and are precipitated when the solution is neutralized to an acidity of about pH 5 to 6.

Both forms of cystine react in a similar manner with chemical reagents, but the derivatives which are formed differ in physical properties and crystal form. Therefore a study of a number of

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<sup>1</sup> Hoffman, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, 1922, xlv, 341-360.

these derivatives was undertaken in the hope that it might throw some light on the structure of the isomeric cystine. A review of the earlier work on the structure of cystine has already been given.<sup>1</sup>

If the now generally accepted formula for cystine as given by Friedmann<sup>2</sup> is correct, the *i*-cystine may be formed by (1) a shifting of some group from one carbon atom to another which would at the same time racemize the compound; or (2) it may be due to a simple racemization of the active form; or (3) the structure of cystine suggests the possibility of an internally compensated meso form, but in so far as we are aware, such a form has never been prepared. The possibility therefore remains that our isomeric cystine may be this meso form.

#### EXPERIMENTAL.

In the attempt to analyze the changes produced by the action of boiling 20 per cent hydrochloric acid on *l*-cystine, a comparative study was made of a number of the organic derivatives of *l*-cystine<sup>3</sup> and of *i*-cystine.<sup>3,4</sup> Certain derivatives of "stone" cystine are included in the study, inasmuch as an unusual opportunity was afforded us to secure authentic samples of stone cystine. The cystine stones were obtained from Dr. E. C. Tennant of Denver, Colorado, who reported<sup>5</sup> the removal of fifteen stones, having a total weight of 73 gm., from the kidney of a woman. Dr. Tennant kindly sent us several gm. of these kidney stones. In a preliminary report<sup>6</sup> we recorded the fact that these stones contained 93 per cent of cystine crystallizing in hexagonal plates and having (a 1 per cent solution of cystine in approximately 0.1 N HCl) an optical rotation of  $[\alpha]_D^{25} = -242.6^\circ$ , the highest optical rotation which has ever been recorded for *l*-cystine.

<sup>1</sup> Friedmann, E., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 1-46.

<sup>2</sup> Hoffman, W. F., MS Thesis, University of Minnesota, 1921.

<sup>3</sup> Since our work was reported at the 1921 meeting of the American Chemical Society, Shipley, G. J., and Sherwin, C. P., *J. Biol. Chem.*, 1923, lv, 671-686, have described a number of new organic derivatives of *l*-cystine, only a few of which were included in our study.

<sup>4</sup> Tennant, E. C., *J. Am. Med. Assn.*, 1923, lxxx, 305-307.

<sup>5</sup> Gortner, R. A., and Hoffman, W. F., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 691-693.

*Dihydrochlorides.*

In the earlier paper<sup>1</sup> we noted that the dihydrochlorides of the *l*- and *i*-cystine show widely different crystal form. This may be best observed by using the procedure described by Denigès,<sup>7</sup> which consists in allowing the hydrochlorides to crystallize out of a concentrated hydrochloric acid solution on a microscope slide. The *l*-cystine hydrochloride and stone cystine hydrochloride both formed long needles, while the *i*-cystine hydrochloride formed large, diamond-shaped crystals. On analysis these were shown to be the dihydrochlorides.

	Theoretical for $C_6H_{12}N_2S_2O_4 \cdot 2 HCl$ .	Found. <i>i</i> -Cystine.	Found. <i>l</i> -Cystine.
Nitrogen . . . . .	8.94	8.72	8.46

*Cysteic Acids.*

*l*-Cysteic Acid.—The method described by Friedmann<sup>2</sup> was used in preparing the cysteic acids. 10 gm. of *l*-cystine were suspended in 75 cc. of water and bromine was slowly added until the solution became slightly yellow. This indicates the presence of free bromine which takes place only when oxidation is complete. The solution was then evaporated to dryness *in vacuo*, and the residue washed by grinding with successive portions of alcohol until the cysteic acid was free of hydrobromic acid. The preparation was recrystallized by solution in water and reprecipitation with alcohol and consisted of a white, powder-like mass of crystals, apparently stellate groups of microscopic needles.

*i*-Cysteic Acid.—The same procedure, as described above, was used in preparing the *i*-cysteic acid. The final product consisted of pure white crystals in the form of fairly large square or rectangular plates. It resembled *l*-cysteic acid in that it did not melt below 245° at which point decomposition began. The compound is optically inactive. It has the same chemical composition as the *l*-cysteic acid but differs particularly in crystal form.

	Theoretical for $C_6H_7O_4NS$ .	Found.
Nitrogen . . . . .	8.29	8.50
Sulfur . . . . .	18.96	18.72

<sup>7</sup> Denigès, G., *J. soc. pharm. Bordeaux*, 1920, lviii, 8-12.

*Phenacyl Esters.*

The method as described by Rather and Reed<sup>8</sup> for the preparation of phenacyl esters of organic acids was used for preparing these esters. This method serves as a very satisfactory means of identifying small amounts of organic acids, due to the definite melting points of the esters.

*Diphenacyl Ester of L-Cysteic Acid.*—This ester was prepared as follows: 1.00 gm. of *l*-cysteic acid and 0.63 gm. of sodium carbonate were dissolved in 12.5 cc. of water. After adding 2.57 gm. of phenacyl bromide and 25 cc. of 95 per cent alcohol, the solution was boiled for 2 hours, under a reflux condenser. It was necessary to add small amounts of 95 per cent alcohol from time to time in order to keep the ester in solution. At the end of 2 hours the solution was filtered while hot, enough water added to make a 40 per cent solution of alcohol, and quickly cooled. The ester crystallized readily from the cooled solution. After recrystallizing from 40 per cent alcohol, the ester melted at 204° (uncorrected). The ester was again recrystallized from 40 per cent alcohol and gave a melting point of 203–204° (uncorrected). The pure product formed small stellate groups of needles or prisms. Only 0.25 gm. of the final product was obtained. No effort was made to purify the second crop of crystals.

*Diphenacyl Ester of D-Cysteic Acid.*—The ester was prepared in the same manner as was the *l*-cysteic ester. 0.35 gm. of this ester was obtained which melted at 210° (uncorrected). The ester crystallized in masses of small plates.

Nitrogen determinations show these to be the diphenacyl esters:

	Theoretical for diphenacyl ester, $C_{15}H_{11}O_7NS$ .	Theoretical for monophenacyl ester, $C_{10}H_7O_5NS$ .	Found. <i>l</i> -Cysteic ester.	Found. <i>D</i> -Cysteic ester.
Nitrogen . . . . .	3.46	4.88	3.47	3.39

The esters appear to be identical in chemical composition but differ in solubility and crystal structure.

We were unable to prepare the monophenacyl esters from the monosodium salts of the *l*- and *D*-cysteic acids. Neither were we able to isolate the phenacyl esters of *l*-cystine and *D*-cystine.

<sup>8</sup> Rather, J. B., and Reed, E. E., *J. Am. Chem. Soc.*, 1919, xli, 75–83.

*Tetracarbethoxy Cystines.*

*Tetracarbethoxy-l-Cystine.*—This compound was prepared by dissolving 2.0 gm. of *l*-cystine in 50 cc. of water containing enough sodium hydroxide to make the sodium salt. After adding 2.2 gm. of ethyl chlorocarbonate to the solution and shaking, it was necessary to add approximately 0.4 gm. more sodium hydroxide to keep the solution alkaline. When the odor of ethyl chlorocarbonate had disappeared, the solution was neutralized with hydrochloric acid and evaporated to dryness *in vacuo*. The residue was extracted with butyl alcohol, which dissolved the derivative but did not dissolve the sodium chloride or any unchanged cystine. The butyl alcohol solution was evaporated to dryness and the residue again extracted with butyl alcohol. After filtering, the butyl alcohol solution was concentrated to a small volume from which the carbethoxy-*l*-cystine separated in a hard crystal mass. The alcohol was decanted off and the compound dried and pulverized. A brown powder was obtained. Due to the deliquescent property of this compound it was very difficult to obtain a dry sample for analysis. The melting point was about 63° (uncorrected). The final yield of the pure compound was 3.55 gm.

*Tetracarbethoxy-i-Cystine.*—This derivative was prepared essentially as described above. When this compound was dried and pulverized, a light gray powder was obtained. This preparation also melted at about 64° (uncorrected). The final yield of the pure product was 3.55 gm.

The compounds analyzed as follows:

	Theoretical for dicarbethoxy derivative, $C_{11}H_{15}O_4S_2N_2$ .	Theoretical for tetracarbethoxy derivative, $C_{13}H_{17}O_6N_2S_2$ .	Found. Tetracarbethoxy- <i>i</i> -cystine.	Tetracarbethoxy- <i>l</i> -cystine.
Nitrogen . . . .	7.29	5.30	5.15	5.20
Sulfur . . . . .	16.69	12.14	12.47	12.14

The analyses show these derivatives to be the tetracarbethoxy derivatives, all of the hydrogens of the amino group being substituted by the carbethoxy groups. The carboxyl groups of cystine are free, as is indicated by the fact that when the compound in an 80 per cent alcoholic solution<sup>9</sup> is titrated with sodium

<sup>9</sup> Foreman, F. W., *Biochem. J.*, 1920, xiv, 451-473.

hydroxide, 2 equivalents of alkali are neutralized by 1 equivalent of the carbethoxy derivative. This is the only compound of cystine which we have prepared in which it was possible to form a tetra derivative on the nitrogen atoms.

*Dibenzoyl Cystines.*

*Dibenzoyl-l-Cystine.*—This derivative was prepared as previously described.<sup>10</sup> The purified product consisted of white silky needles which melted at 181° (uncorrected). Wolf and Rideal<sup>11</sup> state that they obtained a melting point of 189° (uncorrected) for this compound after it had been extracted with benzene to remove the last traces of benzoic acid and benzoyl chloride. We have never obtained a melting point which varied from that originally given, 181° (uncorrected). This is the same melting point as was given by Brenzinger<sup>12</sup> and Neuberg and Mayer,<sup>13</sup> whereas Goldmann and Baumann<sup>14</sup> give 156–158° as the melting point of pure dibenzoyl cystine.

*Dibenzoyl-i-Cystine.*—This derivative was prepared by the same method as was the dibenzoyl-*l*-cystine. When the solution was neutralized with hydrochloric acid, no typical gel<sup>10</sup> was formed as in the case of the dibenzoyl-*l*-cystine, but the compound separated in crystalline form. After purification, the crystals appeared to be diamond-shaped plates or broken fragments of plates. The pure preparation was optically inactive and melted at 168° (uncorrected) but one sample that precipitated from the mother liquor melted at 170° (uncorrected). The melting point was not very sharp; the substance softened at about 120° and did not completely melt below 168°. Neuberg and Mayer<sup>13</sup> prepared the benzoyl derivative of stone cystine for which they report a melting point of 157–159°.

*Dibenzoyl Stone Cystine.*—This compound was prepared exactly as were the compounds noted above, from a sample of *l*-stone cystine.<sup>6</sup> The dibenzoyl derivative crystallized in diamond-

<sup>10</sup> Gortner, R. A., and Hoffman, W. F., *J. Am. Chem. Soc.*, 1921, xliii, 2199–2202.

<sup>11</sup> Wolf, C. G. L., and Rideal, E. K., *Biochem. J.*, 1922, xvi, 548–555.

<sup>12</sup> Brenzinger, K., *Z. physiol. Chem.*, 1892, xvi, 552–588.

<sup>13</sup> Neuberg, C., and Mayer, P., *Z. physiol. Chem.*, 1905, xlv, 472–497.

<sup>14</sup> Goldmann, E., and Baumann, E., *Z. physiol. Chem.*, 1888, xii, 254–261.

shaped plates and melted at 160° (uncorrected). This derivative appears to be identical with that described by Neuberg and Mayer<sup>13</sup> for stone cystine (m.p. 157–159°). It also has the same melting point as that which Goldmann and Baumann<sup>14</sup> report for dibenzoyl-*l*-cystine. Apparently Goldmann and Baumann were working with a sample of cystine prepared from the urine of a patient suffering from cystinurea, consequently their preparation should be classed as stone cystine.

Analysis of the *i*- and stone cystine derivatives gave the following results.

	Theoretical for $C_{22}H_{30}O_4Na_2S_2$ .	Found. Dibenzoyl- <i>i</i> -cystine.	Dibenzoyl stone cystine.
Nitrogen . . . . .	6.25	6.15	6.10
Sulfur . . . . .	14.30	14.16	14.11

From these analyses it is seen that these derivatives have the same chemical composition as the *l*-benzoyl cystine. They differ in physical properties, crystal form, and melting point. It is of particular interest to note that the *i*-cystine which is optically inactive and the stone cystine which has a greater levorotation than does our *l* protein cystine are so nearly alike in physical properties and so widely different from the ordinary dibenzoyl-*l*-cystine.

#### *Cystine Diethyl Ester Dihydrochlorides.*

*l*-Cystine Diethyl Ester Dihydrochloride.—The ester hydrochloride was prepared by suspending 5 gm. of *l*-cystine in a solution of 150 cc. of absolute alcohol containing 10 cc. of concentrated hydrochloric acid and then distilling 250 cc. of absolute alcohol through this solution, thus removing the water added with the hydrochloric acid and also the water formed from the esterification. The reaction was complete after about 30 minutes. The solution was allowed to stand for 20 hours and was then filtered and concentrated to about 50 cc. *in vacuo*. On standing, a part of the ester crystallized and the remainder separated when dry ether was added to the solution. The combined crops of crystals were washed with dry ether, recrystallized from absolute alcohol and ether, and finally dried at 100°. The preparation consisted of 2.8 gm. of very fine, white needles, aggregating in balls or stellate groups (m.p. 177–178° (uncorrected) with decomposition).



*i*-Cystine Diethyl Ester Dihydrochloride.—The compound was prepared in the same manner as the *l*-cystine derivative. It differed, however, in that the reaction appeared to progress much more rapidly and the ester to be much less soluble in absolute alcohol and more soluble in ether. The final product weighed 3.2 gm. and consisted of pure white crystals which appeared to be long narrow prisms (m.p. 169–170° (uncorrected) with decomposition).

The analyses of the two derivatives are:

	Theoretical for $C_{10}H_{20}N_2O_4S_2 + 2HCl$ .	Theoretical for $C_{10}H_{20}N_2O_4S_2 + 3HCl$ .	Found.	
			<i>i</i> -Ester.	<i>l</i> -Ester.
Nitrogen . . . .	7.59	6.91	7.54	7.53
Sulfur . . . . .	17.37	15.81	17.29	17.18
Chlorine . . . .	19.21	26.22	19.28	19.07

These results show that the esters are identical in chemical composition, although they differ in crystal form, melting point, and in solubility. Both of these compounds form the dihydrochloride and not the trihydrochloride. The description of the *l*-cystine diethyl ester hydrochloride prepared by Friedmann<sup>2</sup> agrees very well with our preparation, although his analysis shows that he has a trihydrochloride, melting, with rapid heating, at 185° with decomposition. His higher acid content may be due to adsorbed hydrochloric acid, for he found that cystine formed the dihydrochloride and that cysteine formed the monohydrochloride.

#### *Di-β-Naphthalenesulfoncystines.*

*Di-β-Naphthalenesulfon-l-Cystine.*—This derivative was prepared as follows: 2 gm. of *l*-cystine were dissolved in 25 cc. of normal sodium hydroxide and 9 gm. of  $\beta$ -naphthalenesulfonchloride and 75 cc. of ether were added to this solution. This mixture was shaken for 1 hour, 9 cc. of *N*/1 sodium hydroxide added and the mixture shaken for 1 hour; 9 cc. of *N*/1 sodium hydroxide again added and the shaking continued for another hour; a final 9 cc. of *N*/1 sodium hydroxide were added and, after shaking for another hour, the solution was filtered, the ether was removed, and the aqueous solution was washed with a fresh portion of 50 cc. of ether and then acidified with hydrochloric acid. A heavy, brown, oil-like mass separated. This was insoluble in water. It was re-

crystallized from dilute alcohol. The pure product melted at 203–204° (uncorrected) and consisted of short, microscopic, needle-like crystals or possibly rectangular prisms. The yield of the pure compound was 2.5 gm.

Attempts to prepare  $\beta$ -naphthalenesulfonylcystine according to the method described by Abderhalden,<sup>15</sup> where ammonia is used to dissolve the cystine before the reagent is added, were not successful. In place of the expected derivative we obtained  $\beta$ -naphthalenesulfonamide which melted at 213–214° (uncorrected). The appearance and melting point of our  $\beta$ -naphthalenesulfonamide agree very well with those recorded by Abderhalden for the cystine derivative. His analysis, however, is theoretical for naphthalenesulfonylcystine. The amide was also obtained when attempts were made to prepare the *i*-cystine derivative according to his method. When the procedure was carried out according to Abderhalden's directions but no cystine added, almost theoretical yields of the amide were obtained. We must accordingly conclude that his analyses were in error and that he actually isolated only the sulfonamide.

*Di- $\beta$ -Naphthalenesulfon-*i*-Cystine.*—This compound was prepared by the method described above. When the aqueous solution was acidified with hydrochloric acid, a brown, oily precipitate separated. This was purified by recrystallizing from dilute alcohol. The pure product melted at 215° (uncorrected) with the evolution of gas; yield 2.7 gm.

The analyses show that these derivatives are chemically identical. They both crystallize with 2 molecules of water of crystallization.

	Theoretical for $C_{20}H_{14}O_4N_2S_4$	Theoretical for $C_{20}H_{14}O_4N_2S_4 \cdot 2H_2O$	Found, <i>l</i> derivative.	Found, <i>i</i> derivative.
Sulfur.....	20.68	19.54	18.63	18.29
Nitrogen.....	4.51	4.26	4.23	4.30

### *Cystine Phenylisocyanates.*

*l*-Cystine Phenylisocyanate.—This derivative was prepared by dissolving 2 gm. of *l*-cystine in 24 cc. of water and 18 cc. of *N*/1 sodium hydroxide, and then adding 2 gm. of phenylisocyanate. This mixture was shaken until the odor of the phenylisocyanate

<sup>15</sup> Abderhalden, E., *Z. physiol. Chem.*, 1903, xxxviii, 557–561.

disappeared, filtered, and the filtrate acidified with hydrochloric acid. On acidification, the solution set to a solid crystalline gel which was filtered and purified by recrystallizing from dilute alcohol. The pure compound consisted of very fine needles which melted at 148–149° (uncorrected). A 1 per cent solution of *l*-cystine phenylisocyanate in dilute alcohol set to a rigid gel in a short time. This compound was prepared by Patten<sup>16</sup> but no record of the melting point was given. Neuberg and Mayer<sup>18</sup> have also prepared this compound. They report a melting point of 160° (uncorrected).

*i*-Cystine Phenylisocyanate.—The derivative was prepared in the same manner as was the *l* compound. A difference was noted in that this compound did not form a gel as easily as did the *l* derivative. This preparation consisted of very fine, long, silky, colorless needles which melted at 181° (uncorrected).

(Stone) Cystine Phenylisocyanate.—This was prepared from a sample of stone cystine.<sup>6</sup> It crystallized from dilute alcohol in thin, irregular plates and melted after recrystallization at 132–133° (uncorrected). Neuberg and Mayer report the melting point of stone cystine phenylisocyanate as 170–172° and the crystal form as a colorless microcrystalline mass.

The analyses show that although these three compounds differ in physical properties and crystalline structure, nevertheless they have the same chemical composition.

	Theoretical for $C_{20}H_{22}O_4N_4S_2$ .	<i>i</i> derivative.	Found. <i>l</i> derivative.	Stone derivative.
Nitrogen . . . . .	11.71	11.71	11.58	11.72
Sulfur . . . . .	13.41	13.47	13.48	13.27
Carbon . . . . .	50.17	50.46		
Hydrogen . . . . .	4.64	5.05		

### *Cystine Phenylhydantoins.*

*l*-Cystine Phenylhydantoin.—The compound was prepared by boiling 2 gm. of *l*-cystine phenylisocyanate with 150 cc. of 10 per cent hydrochloric acid for 5 minutes. A clear solution resulted and on cooling, a white precipitate separated. The precipitate was filtered off and recrystallized from alcohol. The pure preparation consisted of white needles which melted at 122–123° (uncorrected).

<sup>16</sup> Patten, A. J., *Z. physiol. Chem.*, 1903, xxxix, 350–355.

This compound was first prepared by Patten<sup>16</sup> and later by Shiple and Sherwin,<sup>4</sup> both of whom report a melting point of 117° (uncorrected).

*i*-Cystine Phenylhydantoin.—It was impossible to prepare this compound by boiling the *i*-cystine phenylisocyanate in 10 per cent hydrochloric acid for 5 minutes as in the case of the *l*-cystine derivative. At the end of 5 minutes no change had occurred, the isocyanate being recovered practically quantitatively, and at the end of 35 minutes boiling only a part of the phenylisocyanate had changed to the hydantoin. After recrystallization from alcohol the pure compound formed small tyrosine-like crystals which melted at 166° (uncorrected). The analyses show this to be identical in composition with *l*-cystine phenylhydantoin.

Stone Cystine Phenylhydantoin.—This was prepared from the stone cystine phenylisocyanate by boiling with 10 per cent hydrochloric acid for 5 minutes. The phenylisocyanate dissolved completely and on cooling the hydantoin separated. It was recrystallized from dilute alcohol and was obtained in the form of stellate groups of needles which begin to soften at 105° and melt sharply at 112° (uncorrected). Neuberg and Mayer<sup>12</sup> report that they were unable to prepare the phenylhydantoin of their stone cystine, although the corresponding derivative of their protein cystine was easily obtained.

	Theoretical for $C_{20}H_{18}O_4N_4S_2$	<i>i</i> -Cystine phenylhydantoin.	Found. Stone cystine phenylhydantoin.
Carbon .....	54.25	52.95	
Hydrogen .....	4.10	3.91	
Nitrogen .....	12.67		12.71
Sulfur .....	14.50	14.48	

### Cysteines.

*l*-Cysteine.—The compound was prepared by the reduction of *l*-cystine with tin and hydrochloric acid. After complete reduction the solution of cysteine hydrochloride was filtered free of undissolved tin and evaporated to dryness. The residue was then taken up with water and the tin removed by means of hydrogen sulfide. When the tin-free solution was evaporated to dryness, the cysteine hydrochloride was obtained. Cysteine was obtained by exactly neutralizing the alcoholic solution of the

cysteine hydrochloride with ammonium hydroxide. A flocculent precipitate was formed which appeared to be microcrystalline. After filtering, washing with alcohol, and drying, the compound appeared to consist of dust-like particles of no definite crystal form. It gave the following color tests for cysteine: With sodium hydroxide and sodium nitroprusside, a violet-pink color; with copper sulfate, a quickly disappearing blue color; with ferric chloride, a purple color which soon disappeared.

*i-Cysteine.*—The *i*-cysteine was prepared in the same manner. When precipitated with ammonium hydroxide, a heavy, crystalline precipitate was formed, the crystals appearing to be flat irregular plates. They gave the same color reactions as did *l*-cysteine. The striking difference noted between the two compounds was the ease with which the sulfur was split off from the *l*-cysteine with sodium hydroxide, as compared with *i*-cysteine. It was necessary to boil the latter some time with strong hydroxide before a test for unoxidized sulfur was obtained.

#### *Benzyl Cysteines.*

*Benzyl-l-Cysteine.*—This derivative was prepared as follows: 3 gm. of *l*-cysteine hydrochloride were dissolved in a small amount of water and 2 gm. of sodium hydroxide and 3 gm. of benzyl bromide were added to this solution. After shaking this mixture for 1 hour, the unchanged benzyl bromide and benzyl alcohol were removed by extraction with ether and the aqueous solution acidified with acetic acid. This was then concentrated to a small volume from which the benzyl cysteine crystallized. The crude product was purified by recrystallizing from hot water. The purified preparation melted at 213° (uncorrected) with browning and the evolution of gas. The crystals formed stellate groups of needles.

	Theoretical.	Found.
Nitrogen .....	6.63	6.70

*Benzyl-i-Cysteine.*—The compound was prepared by the same method as was used for the *l* derivative. The pure product was snow-white and consisted of 2.1 gm. of elongated, flat plates with pointed ends. This preparation melted at 190° (uncorrected) with browning and the evolution of gas. It was necessary to heat

very slowly in order to obtain a constant melting point inasmuch as the material loses water of crystallization during the heating process.

Dried at 100°, the benzyl *i*-cysteine analyzed as follows:

	Theoretical for $C_{10}H_{13}O_2SN$ .	Theoretical for $C_{10}H_{13}O_2SN \cdot 2H_2O$ .	Found.
Carbon .....	56.85	48.57	49.08
Hydrogen .....	6.16	6.88	6.82
Nitrogen .....	6.63	5.67	5.65
Sulfur .....	15.19	12.98	12.73

The benzyl *i*-cysteine contains 2 molecules of water of crystallization, while the *l* derivative contains none. The melting point and crystal structure are also different.

#### *Attempts to Prepare Taurine from Cystic Acid.*

The structural formula for cystine is based largely on Friedmann's<sup>2</sup> observation that cystic acid can be converted into taurine. Friedmann heated 2 gm. of cystic acid in the presence of 15 cc. of water in a bomb tube, raising the temperature during 2 hours to 235° and maintaining the temperature for a further 2 hours between 235° and 240°. Carbon dioxide was split off and taurine resulted. Although we have made a number of attempts, we have been wholly unable to duplicate this experiment. In our later experiments a much longer period of heating than that recommended by Friedmann was employed but the cystic acid was recovered unchanged. A typical protocol of such an experiment follows:

2 gm. of *l*-cystic acid and 15 cc. of water were sealed in a bomb tube and the time and temperatures of heating were as follows:

	Temperature. °C.
11.00 a.m., began heating.	
11.30 " .....	100
12.30 p.m. ....	180
1.30 " .....	230
2.30 " .....	235
3.30 " .....	233
5.00 " .....	235
6.30 " heating discontinued .....	235

The tubes were allowed to cool and were opened the next morning. There was slight pressure and some mercaptan odor. The solutions were filtered, concentrated, and the solid recrystallized from dilute alcohol. The crystal form was characteristic of *l*-cysteic acid. The substance titrated, in 85 per cent alcohol with phenolphthalein as an indicator, as a dibasic acid, the titration values being theoretical for cysteic acid.

Similar attempts to prepare taurine from the *i*-cysteic acid resulted in recovering unchanged *i*-cysteic acid. This recovered material titrated as a dibasic acid.

Analyses of material from bomb tubes were as follows:

	Theoretical for cysteic acid, $C_3H_7O_4NS$ .	Theoretical for taurine, $C_2H_7O_4NS$ .	Found.	
			<i>l</i> -Cysteic acid.	<i>i</i> -Cysteic acid.
Carbon .....	21.30	19.18		21.33
Hydrogen .....	4.14	5.64		4.50
Nitrogen .....	8.29	11.20	8.31	8.30
Sulfur .....	18.96	25.63		19.63

If cysteic acid can be converted into taurine by heating in a bomb tube according to Friedmann's directions, there is certainly some unknown factor involved which is not evident in his directions. Our cysteic acid was not prepared through the copper salt as was Friedmann's preparation. It may be that copper catalyzes the reaction. We have not tested this possibility. The point is an important one, for, as noted above, the structural proof of cystine is based upon this reaction which in our hands has failed in at least ten separate attempts.

#### *Attempts to Prepare Dithiodilactic Acid from l-Cystine and i-Cystine.*

Friedmann<sup>2</sup> reports the preparation of these compounds from cystine. All of our efforts to repeat his experiments have resulted in failure.

#### *Attempts to Resolve i-Cystine into Optically Active Isomers.*

*Biological Method.*—Attempts were made to utilize *Aspergillus niger* as a biological agent to determine whether or not *i*-cystine is a racemic mixture of *d*- and *l*-cystine. A solution, containing the necessary mineral salts and 7.5 gm. of *l*-cystine dissolved in dilute ammonia and slightly acidified with phosphoric acid, was

prepared and inoculated with spores of *Aspergillus niger*. 3 days later the growth was pronounced and the culture was sporulating. The culture was still growing well at the end of 22 days, when it was made slightly alkaline with ammonia, filtered, the filtrate decolorized with norit and acidified with acetic acid. The precipitated cystine was filtered off, washed, and dried. It showed a specific rotation  $[\alpha]_D = -216^\circ$ .

A similar experiment, using *i*-cystine had ceased growth at the end of the 22 day period. The clarified filtrate from the mycelium showed no optical activity and cystine which was isolated from this filtrate was likewise optically inactive. No resolution was accordingly effected.

*Chemical Method.*—Attempts were made to resolve *i*-cystine by preparing the cinchonidine and brucine salts of benzoyl *i*-cystine. The salts were readily formed by heating the components in 50 per cent alcohol. A fractionation of the resulting product according to its solubility in alcohol yielded three fractions but, when these fractions were decomposed and the benzoyl *i*-cystine recovered, there was no optical activity in any of the preparations. Similar failure to secure resolution resulted from the use of *i*-cystine and *d*-camphoric acid, and of *i*-cysteic acid and cinchonidine. In the experiments involving *i*-cysteic acid and cinchonidine the salt, which was obtained, could be separated into fractions which differed markedly in solubility in absolute alcohol. No attempt was made to analyze these fractions but, when the cysteic acid was prepared from them, it was wholly inactive and identical with the starting material.

#### CONCLUSIONS.

A number of derivatives of *l*-cystine and of *i*-cystine (prepared from *l*-cystine by boiling with 20 per cent hydrochloric acid) have been prepared. In each instance both forms of cystine form derivatives having the same empirical formula<sup>17</sup> but in no instance have we found them to be alike in melting point and in but few instances to be identical in crystal form. Attempts to resolve the *i*-cystine into optically active components have failed.

This study throws no light on the change which takes place

<sup>17</sup> In one or two instances there is a difference in water of crystallization.



when cystine is boiled with a strong acid. It does indicate, however, that the change is a profound one and is reflected in the physical properties of derivatives prepared from the altered cystine.

Our failure to resolve the *i*-cystine leads us to believe that it may not be racemic cystine but rather that it may be an internally compensated form.

There are many discrepancies in the literature in regard to the physical properties of derivatives of cystine. This is particularly true when we compare derivatives of *l*-cystine and of stone cystine. Our data for stone cystine and *i*-cystine add additional discrepancies. The fact that our stone cystine<sup>6</sup> had an optical rotation of  $[\alpha]_D^{20} = -242.6^\circ$ , which is approximately  $20^\circ$  higher than the usually accepted value for *l*-cystine, indicates, we believe, that the *l*-cystine, as usually prepared from protein by acid hydrolysis, may be appreciably contaminated with our *i*-cystine. It is possible that such contamination may account for some of the discrepancies which have been recorded.

## STUDIES ON ENZYME ACTION.

### **XL. TIME CHANGES IN ESTER-HYDROLYZING ACTIONS OF EXTRACTS OF WHOLE RATS OF DIFFERENT AGES.**

BY HELEN MILLER NOYES AND K. GEORGE FALK.

(From the Harriman Research Laboratory, The Roosevelt Hospital,  
New York.)

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#### INTRODUCTION.

In a series of papers published in the past 5 years, a systematic study of certain enzyme actions of normal and abnormal tissues was presented. This study is still in progress, but in the course of it, certain results have been obtained which appear to offer a new mode of attack for following the chemical changes which may occur during the life process. The work previously presented includes the enzyme action of a given tissue preparation at a definite period in the life process. By the combination of a number of such periods of different individuals, the course of the change during the life cycle of an individual of the given species as deduced from the states of the separate individuals, has been developed. This is the customary method of following the chemical changes which take place, or which are assumed to take place, in living organisms.

The method which is to be described here aims to follow *in vitro* in materials obtained from the living organism some of the changes which occur in life processes. Chemical change with passage of time is involved. The belief that the method gives true indications *in vitro* paralleling the changes occurring *in vivo* is based upon the comparison of the results in the two sets of cases at various stages.

The general idea underlying the method is as follows: Tissues obtained from an animal immediately upon its death show certain enzyme actions. For reasons which have been outlined on

several occasions, a line of studies in this laboratory has involved the use of ester-hydrolyzing enzymes and the comparison of the action of a given tissue on a number of esters. For practical reasons extracts of the tissues were used. The enzymes in abnormal growths such as tumors would be expected to be different from those in normal growth. In the ordinary course of growth and development from the embryo to the adult, changes in enzyme character and content would be expected. Such differences in the ester-hydrolyzing enzymes have been found for tumors in comparison with normal tissues,<sup>1</sup> for whole embryos as compared with the adult animal for rats,<sup>2</sup> for mice,<sup>3</sup> for a number of tissues of the embryo rabbit as compared with the tissues of the adult rabbit, and for some human tissues.<sup>4</sup> In considering a possible mechanism of the chemical changes which result in these enzyme differences, it may be imagined that the cell materials in some way are changed. There is a constant interchange of matter within and without the cells, so called nourishment being supplied, the products of metabolism removed, etc. It is probable that the substances present in the cell undergo definite changes in the transformations to which reference has been made, in which the substances added or removed may or may not be involved. If such definite changes take place in substances present in the living matter during the life process whether in normal or abnormal growth, it is possible that similar changes take place under suitable conditions in these substances when removed from the living matter. The ester-hydrolyzing actions which have been described have been shown to differentiate between different tissues and between various stages of growth. It appears that, at times, they show relations and differences

<sup>1</sup> Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183, 213. Noyes, H. M., and Falk, K. G., *J. Biol. Chem.*, 1924-25, lxii, 687. Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924-25, lxii, 697. Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Cancer Research*, 1925, ix, 105. Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1925, ix, 129.

<sup>2</sup> Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925-26, viii, 75.

<sup>3</sup> Falk, K. G., and Noyes, H. M., *J. Gen. Physiol.*, 1926-27, x, 359.

<sup>4</sup> Noyes, H. M., Falk, K. G., and Baumann, E. J., *J. Gen. Physiol.*, 1925-26, ix, 651.

which do not appear to be shown by any other chemical method at present available. These enzyme actions were therefore used to test the materials (tumor as well as normal tissues) obtained from living organisms. The extracts of these materials, which when freshly prepared showed characteristic enzyme pictures, were tested periodically after standing at room temperatures under antiseptic conditions for different lengths of time, in some cases up to 2 years. Definite changes in the amounts and types of the enzyme actions were observed. That enzyme actions change under such conditions is not surprising. In fact most enzymes are assumed to lose their activity gradually under these conditions. Also it may not be considered unusual that different enzyme actions change at different rates, or that the same enzyme action (or group of actions) on different substrates shows different orders of stability toward these substrates. The fact that order can be evolved out of these changes, that by certain methods of treating the experimental results, systematic changes appear clearly, is the new feature that it is desired to emphasize in this and the following papers. The feature of the results to be described, therefore, is the fact that the "pictures" or types of the actions change, and change in such ways that types of actions characteristic of certain other materials are obtained. If it were not for this parallelism to certain physiological and pathological changes, the conclusions to be drawn from such changes in enzyme actions would be incomplete, but the fact that this parallelism has occurred in a number of cases and with considerable regularity, justifies the presentation of these results. It may appear remarkable that the enzyme changes in the extracts parallel even remotely enzyme changes in tissues in different phases of growth considering the complex nature of the latter, but the facts which will be presented indicate that this is the case.

This problem may also be considered from another point of view. The extracts of various tissues and tumors showed enzyme actions, which, when studied in the manner developed in previous papers, may be said to characterize them within reasonable limits. The changes which these extracts, and therefore presumably the enzymes contained in them, undergo under different conditions are naturally of interest, since in this way it is possible that light may be thrown on changes which occur in life processes, and

possibly in the changes which occur when so-called normal growth is transformed into the so-called abnormal growths grouped under tumors, both benign and malignant. The first changes to be studied in the extracts would be those which occur spontaneously without the addition of foreign substances to modify the enzyme actions.

In this and the following papers, the results obtained with extracts of whole rats, of whole mice, of fibromyoma of the human uterus, of human uterine muscle, and of a number of rabbit tissues, will be presented.

#### EXPERIMENTAL.

##### *Methods.*

The extracts of whole rats of definite ages whose ester-hydrolyzing actions were described previously<sup>2</sup> were allowed to stand at room temperature which ranged between the extremes of 10 and 30°C., and the enzyme actions tested at intervals. The extracts were diluted to give the same concentrations as in the initial tests and brought to pH 7.0 in the cases where any change had occurred. The conditions for the repeating tests were kept as uniform and as nearly like the original determinations of enzyme actions as possible. An excess of toluene was present in the extracts at all times. The concentrations of the extracts in all the experiments, except where otherwise stated, corresponded to 26.7 mg. of original material per cc. of extract tested.

##### *Results.*

The enzyme actions of a number of the rat extracts were determined at intervals up to 23 months. The older rats were large enough to furnish sufficient material for such an extended study, but with the younger rats this was not feasible and the results with these may be considered in part to be incomplete. Even so, it is evident that a large mass of material is available, which cannot be presented in detail. The data will therefore be grouped as far as possible and, when necessary, representative results chosen to bring out the relations. It is necessary to present both the absolute enzyme actions and the relative actions or pictures.

It was shown in the earlier paper that the absolute values and

the pictures of the enzyme actions changed in a characteristic manner with increasing age of the rats. This must be borne in mind in considering the changes on standing of the different extracts.

The results obtained with the extracts which were tested after the longest time intervals will first be given. These include rats between the ages of 545 and 1110 days at the time of killing. The actions after standing about 300 days reached values which changed only very slowly thereafter. Not only were constant or nearly constant values obtained, but the values for the different

TABLE I.

*Average Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Extracts of Whole Rats which Have Stood the Indicated Number of Days.*

Ester.	Absolute actions. Extracts after standing days (average).			
	321	406	542	667
Phenyl acetate.....	1.39	1.41	1.21	0.90
Glyceryl triacetate.....	0.79	0.75	0.72	0.59
Methyl butyrate.....	0.33	0.29	0.30	0.22
Benzyl acetate.....	0.41	0.31	0.28	0.23
Ethyl ".....	0.14	0.14	0.17	0.10
Methyl ".....	0.19	0.18	0.20	0.16
Ethyl butyrate.....	0.28	0.30	0.23	0.17
Methyl benzoate.....	0.09	0.07	0.08	0.06
Ethyl ".....	0.06	0.05	0.06	0.03
Isobutyl acetate.....	0.20	0.21	0.20	0.16

extracts varied to only small extents, irregularly, about average values. These averages will therefore be given in Table I.

The times of standing of the extracts which are included in the table with the average of 321 days ranged from 315 to 332 days; those whose average was 406 days, from 398 to 419 days; those whose average was 542 days, from 534 to 555 days, and those whose average was 667 days, from 659 to 676 days. Extracts of eight rats were used for the first three time intervals, of seven rats for the last time interval, the same extracts being tested after the different time intervals. It was shown in the earlier paper that the curves or pictures of the extracts of the oldest rats when tested immediately differed in certain definite respects from those of the

adult rats not so old. The actions of the extracts of both sets of rats after standing 300 days were essentially the same. It will be shown later in this paper that the rates of change of the enzyme actions of these extracts are different depending upon the ages of the rats when killed. The point to be emphasized in the present connection is that the extracts of these adult and old rats reached a common constant standard of ester-hydrolyzing actions after a certain length of time and that these values did

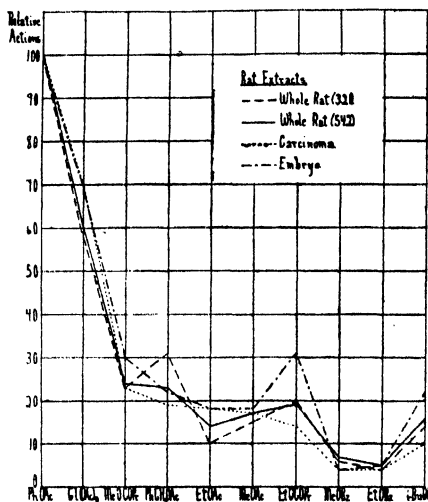


FIG. 1. Relative ester-hydrolyzing actions of whole rat extracts after standing 321 days and 542 days (averages of eight extracts of adult rats for each curve), of the Flexner-Jobling rat carcinoma average, and of the whole rat embryo (averages of embryos aged 3 days before birth to 1 day before birth). The four curves show essentially the same picture.

not change to any marked extent even after the extracts had stood almost 1 year longer. These values were reached after 300 days or possibly less and refer, as shown, to the absolute actions and therefore also to the relative actions or pictures calculated from these. It is true that the actions were small on all the esters except phenyl acetate and glyceryl triacetate.

In Fig. 1 are shown the curves of the relative actions calculated from the average actions of the extracts after standing 321 and

542 days, calculated from the results of Table I, and the curves of the relative actions of the Flexner-Jobling rat carcinoma and of the averages of five rat embryos taken from the earlier papers. Aside from somewhat larger actions on methyl and ethyl butyrates and isobutyl acetate of the rat embryos (whose ages as far as could be told ranged from 3 days before birth to 1 day before birth) the four curves coincide very closely. While the absolute actions of the extracts of the Flexner-Jobling rat carcinoma and of the rat embryos for corresponding concentrations are of the same order of magnitude when tested as soon as possible after preparation of the extracts, they are considerably larger (2 or 3 times) than the absolute action of the extracts of adult and old rats which have stood 300 days. It is very probable that the absolute actions of extracts of the rat embryos and of the Flexner-Jobling rat carcinoma would decrease on standing long periods of time, while the relative actions or pictures would remain practically unchanged. Unfortunately, the amounts of extracts available with the rat embryos did not permit of such extended series of tests, while at the time the rat carcinoma was studied, the development of the work in the indicated direction was not foreseen.

Although it is not practicable to present all the detailed experimental results which have been obtained, it is, however, essential to present enough of the material to make clear the changes in enzyme actions which occur. The results in Table II show the actions of the extracts on glyceryl triacetate and on methyl butyrate immediately upon preparation and after standing different lengths of time. These esters were chosen as representative of the general behavior.

The results given in Table II show that the rates of change of the enzyme actions are different for the rats of different ages and that for any one age the rates of change are different on the two esters. This will make clear the difficulty of presenting the experimental results. The possible variables include the ages of the rats, the lengths of time of standing of the extracts, and the varying actions on ten different esters. The data presented in Table II show that for the younger rats the actions either remained constant or increased as the extracts were kept for short time periods (up to about 30 days).



TABLE II.

*Absolute Actions in Tenths of Milli-Equivalents of Glyceryl Triacetate and of Methyl Butyrate Hydrolyzed by Extracts of Whole Rats of Indicated Ages after Different Periods of Time.*

Age of rat.	Extract standing.	Action on $\text{GI}(\text{OAc})_h$ .	Action on $\text{MeOCOPr}$ .	Age of rat.	Extract standing.	Action on $\text{GI}(\text{OAc})_h$ .	Action on $\text{MeOCOPr}$ .
days	days			days	days		
3½	0	2.72	1.31	24(A)	0	3.45	4.25
	8	2.94	1.73		13	2.47	1.07
	16	2.92	1.68		20	2.38	0.89
4	0	2.55	1.81	24(B)	0	3.09	3.90
	12	2.64	1.96		13	2.38	1.16
	22	2.58	1.84		20	2.25	0.81
5	0	2.22	1.41		34	2.19	0.89
	6	2.69	1.78		139	1.52	0.49
8	0	2.64	1.94	25	0	2.66	2.69
	12	2.60	1.93		10	2.34	1.82
	22	2.68	1.95		22	2.13	0.68
	29	2.57	1.85		39	2.05	0.57
11	0	2.21	1.94	27(A)	0	3.09	3.14
	6	2.50	2.01		8	2.59	1.13
12	0	2.81	2.15		15	2.43	0.87
	14	2.96	2.13		24	2.22	0.80
	119	1.76	0.72		34	2.22	0.70
16	0	1.71	0.63	27(B)	0	3.02	3.29
	168	1.58	0.54		8	2.36	1.01
17	0	2.99	3.09		15	2.31	0.80
	14	2.99	2.93		24	2.36	0.83
					34	2.11	0.66
22(A)	0	3.71	2.62	28	0	2.66	2.91
	3	3.35	3.55		6	2.51	2.71
	9	3.28	3.20		17	2.11	1.86
22(B)	0	2.75	2.76		26	1.73	0.89
	9	2.45	2.35		33	1.65	0.61
	23	2.02	0.73		39	1.76	0.54
	29	1.95	0.70		45	1.51	0.42
	147	1.31	0.13	30	0	3.32	3.34
22(C)	0	2.48	2.40		7	2.91	2.24
	12	2.18	1.06		17	2.45	1.08
	20	2.08	0.61		27	2.39	0.83
	26	1.93	0.53		42	2.23	0.72

TABLE II—Continued.

Age of rat.	Extract standing.	Action on $\text{Gl}(\text{OAc})_2$ .	Action on $\text{MeOCOPr}$ .	Age of rat.	Extract standing.	Action on $\text{Gl}(\text{OAc})_2$ .	Action on $\text{MeOCOPr}$ .
<i>days</i>	<i>days</i>			<i>days</i>	<i>days</i>		
30(A)	0	3.10	3.85	100	0	2.70	5.61
	9	2.76	3.28		13	2.42	4.60
	23	2.25	1.02		25	2.13	4.10
	29	2.10	0.86		32	2.13	4.68
30(B)	0	3.58	4.33		52	1.77	1.01
	7	2.76	2.20		76	1.68	0.83
	17	2.37	1.23		84	1.69	0.83
					357	0.90	0.36
40(A)	0	2.56	3.80		444	0.87	0.33
	7	2.11	1.85	126	0	2.34	5.07
	15	1.83	0.62		12	2.31	4.86
	22	1.97	0.58		19	2.26	4.69
	32	1.76	0.53		39	1.65	2.02
	42	1.88	0.62		63	1.49	1.11
	49	1.72	0.47		71	1.38	0.96
	76	1.68	0.40		344	0.77	0.31
40(B)	0	2.99	3.79	189	0	2.48	4.71
	7	2.54	2.78		12	2.09	2.56
	15	2.25	2.04		21	1.74	2.14
71(A)	0	2.85	3.76		39	1.46	1.28
	9	2.57	2.24		67	1.27	1.03
	19	2.20	0.98		91	1.23	0.88
	44	2.06	0.56		117	1.26	0.82
	53	2.03	0.58		207	1.04	0.54
	62	1.88	0.45		344	0.80	0.45
71(B)	0	3.02	3.55	285	0	1.97	4.28
	10	2.39	1.77		10	2.06	4.67
	35	2.17	0.71		21	1.67	3.26
	44	2.16	0.70		44	1.37	1.48
	53	2.07	0.65		62	1.23	1.08
	63	1.83	0.56		100	1.14	0.79
89	0	2.67	3.88		202	0.96	0.49
	27	1.81	0.62		339	0.51	0.19
	35	1.81	0.53				
	47	1.81	0.57				
	55	1.77	0.51				

Age of rat.	Extract standing.	Action on $\text{Cl(OAc).}$	Action on $\text{MeOCoPr.}$	Age of rat.	Extract standing.	Action on $\text{Cl(OAc).}$	Action on $\text{MeOCoPr.}$
<i>days</i>	<i>days</i>			<i>days</i>	<i>days</i>		
367	0	2.07	3.41	748	0	2.42	4.04
	10	2.04	3.78		9	2.42	3.55
	21	1.49	2.32		27	1.88	1.52
	44	1.34	1.26		55	1.65	0.93
	64	1.24	0.96		93	1.60	0.82
	105	1.21	0.76		195	1.21	0.46
	202	1.00	0.52		332	0.78	0.40
	339	0.67	0.38		419	0.73	0.29
545				913	552	0.79	0.34
					676	0.59	0.23
	0	2.19	4.16		0	2.35	3.42
	28	1.69	3.02		23	2.26	3.05
	43	1.53	2.31		43	1.86	2.26
	84	1.45	1.42		84	1.53	1.09
	183	1.05	0.63		183	1.17	0.55
	318	0.85	0.53		318	0.88	0.39
	405	0.83	0.50		405	0.88	0.41
	541	0.77	0.44		541	0.82	0.32
555	665	0.61	0.28	994	673	0.65	0.27
					0	2.10	2.93
	0	2.81	3.20		28	1.65	1.10
	30	2.07	2.16		41	1.49	0.76
	38	1.62	1.67		82	1.47	0.55
	84	1.45	0.99		181	0.99	0.26
	177	1.05	0.47		320	0.75	0.15
	315	0.87	0.42		403	0.68	0.15
	398	0.77	0.32		539	0.61	0.13
	534	0.77	0.41	1055	671	0.52	0.07
740	661	0.63	0.34		0	2.07	2.39
					28	1.61	1.16
	0	2.58	4.07		41	1.38	0.92
	30	1.86	2.35		82	1.30	0.70
	38	1.80	1.99		181	0.89	0.31
	84	1.39	0.84		320	0.61	0.22
	177	1.00	0.46		403	0.61	0.20
	316	0.79	0.32		539	0.55	0.20
	399	0.71	0.25		663	0.40	0.10
	535	0.73	0.28	1110	0	2.28	2.41
	659	0.58	0.24		9	1.85	1.20
					27	1.62	0.55
					55	1.48	0.52
					93	1.41	0.50
					195	1.21	0.37
					332	0.82	0.19
					419	0.76	0.21
					555	0.72	0.24

On longer standing the actions decreased, those on methyl butyrate more rapidly than those on glyceryl triacetate. Beginning with the rats about 22 days old no increases or constancies were observable, and the decreases, especially on methyl butyrate, began after much shorter periods of standing and were much more rapid. These rates of change appeared to hold until the rat ages of 100 days, when the decreases became considerably less rapid on standing and in several cases (285 and 367 day old rats) showed markedly increased actions on the methyl butyrate. For the oldest rats, the rates of decrease became more rapid again.

The results given in Fig. 1 showed that the extracts finally gave ester-hydrolyzing actions similar in type to the rat embryo and the Flexner-Jobling rat carcinoma. These results showed fairly constant absolute actions as well as constant types after 300 days for all the rats of different ages whose extracts were tested. In view of the different rates of change on standing of the actions on the esters by the extracts of rats of different ages shown in Table II, it is of interest to determine the lengths of time of standing when the various extracts definitely reached the final picture, embryonic or Flexner-Jobling rat carcinoma, in character. In determining these lengths of time, only the pictures of the actions are considered; in practically all cases, the absolute actions of the extracts continued to decrease for various lengths of time without however changing the types of the curves or pictures of the actions.

The lengths of time of standing when the types of actions reached the embryonic picture are given in Table III for all the extracts tested. Because of the different intervals of time used, there is naturally a certain error in the times when the changes are stated to be complete.

The results given in Table III fall into four groups. Up to the age of 17 days the enzyme actions of the extracts either increased or remained practically constant for reasonable periods of time. In one case the change to embryo or rat tumor type had occurred in something less than 119 days, in the other cases in the shorter periods such a change in type had not occurred. Between the ages of 22 and 89 days the reversion to embryonic or rat tumor type occurred with the extracts standing 3 weeks or less. This reversion of the extracts with rats between the ages

of 100 and 913 days when killed took considerably longer times. Again for the three oldest rats, the reversion to the embryonic or rat tumor types took a shorter time, 4 weeks or less.

The extracts of the youngest and of the adult rats seem to be more stable as far as the ester-hydrolyzing actions are concerned in the time that it takes for these extracts to become definitely of embryonic or rat tumor type. The extracts of the rats killed at or some time after the weaning period and before becoming adult as well as of the oldest rats are less stable in that the final

TABLE III.

*Periods Required for the Extracts of Whole Rats of Different Ages to Reach the Embryonic Picture of Ester-Hydrolyzing Actions.*

Age of rat when killed.	Period required to reach embryonic picture.	Age of rat when killed.	Period required to reach embryonic picture.	Age of rat when killed.	Period required to reach embryonic picture.
days	days	days	days	days	days
3½	No change. (16)	27	8	126	39-63
4	Increase. (22)	27	8	189	39
5	" (6)	28	17-26	285	44-62
8	No change. (29)	30	7-17	367	44
11	Increase. (6)	30	23	545	84-183
12	Less than 119.	30	7-17	555	84
17	No change. (14)	40	15	740	84
22	23	40	7-15	748	27-55
22	12-20	71	10-35	913	84
24	13-20	71	19	994	28
24	13-20	89	27	1055	28-41
25	10-22	100	52	1110	9-27

type or picture of the enzyme actions is reached in considerably shorter time periods.

Since it is obviously impossible to present the complete changes in the enzyme actions here, results for extracts of six rats only will be given. Those chosen are representative of the different kinds of changes which were found. These results are presented in Fig. 2. The absolute ester-hydrolyzing actions as found are given for the extracts of the six rats immediately upon preparation of the extracts and after standing the indicated lengths of time. These periods of time are given only up to 300 to 400 days, since it was shown in Table I and Fig. 1 that both the absolute and

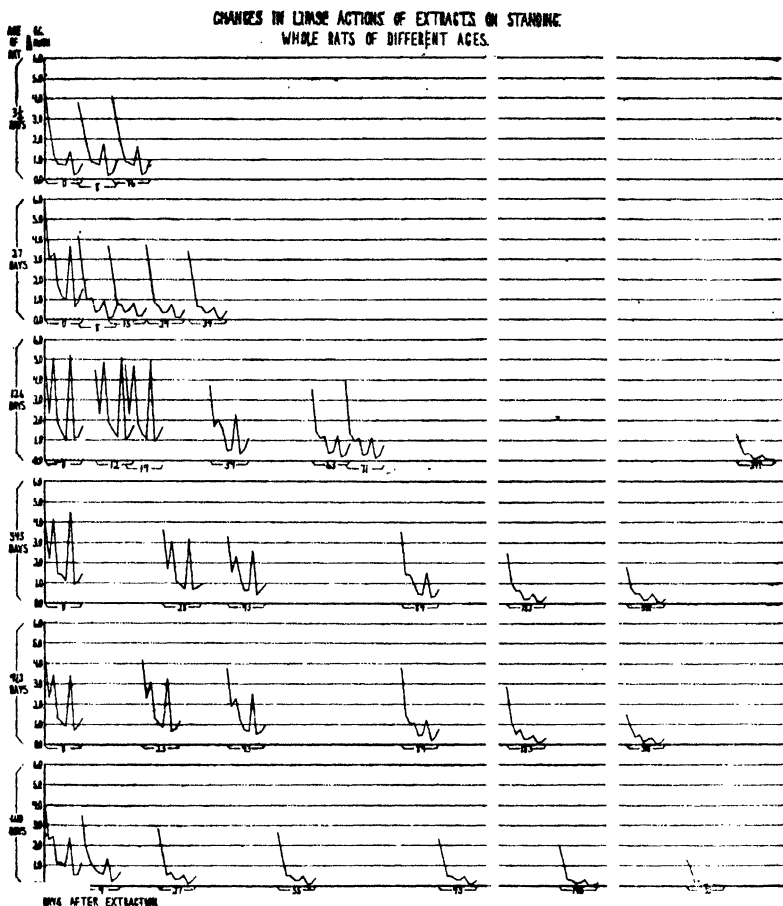


FIG. 2. Absolute hydrolyzing actions on the ten esters of extracts of six whole rats of selected ages immediately upon preparation and after standing different lengths of time. The absolute actions are given as ordinates for each of the six rat extracts separately. Two sets of units are given as abscissas. The actions on the ten esters arranged in the same order along the abscissa axis as in the other plots in this study are grouped for each extract at the time the actions were determined. The number of days after extraction when the actions were determined are also shown along this axis according to scale. To avoid too large a figure, two breaks in the lines denoting the abscissa indicate greater elapsed time, the first break 77 days, the second 114 days. Each curve, therefore, shows the absolute actions of the extract of the whole rat of the indicated age, determined after the number of days the extract had been allowed to stand as stated below the given curve. The different rates of changes of the extracts are explained in the text.

relative actions did not change to any marked extent on longer standing with these extracts.

The facts brought out in Fig. 2 may be summarized as follows: The absolute actions of the youngest rat (3½ days) increased slightly, and the type, approximately that of rat embryos and the Flexner-Jobling rat carcinoma, did not change for the comparatively short time interval the extract was allowed to stand. The remaining extracts shown in the figure showed decreased actions on standing, the types changing because the actions on the different esters decreased at different rates, until all the ages showed identical final types as brought out in Table I. The actions of the 27 day and 1110 day rats decreased much more rapidly than the actions of the extracts of the three adult rats, reaching the final embryonic type of action after comparatively short times of standing. It is true that the adult rats showing adult types of actions had to undergo greater changes to reach the embryonic type than did the young rat and the oldest rat, but even this does not account for the difference in times required for the typical rat actions to be transformed into the embryonic or rat tumor types of actions.

#### DISCUSSION.

The general problem under investigation was outlined in the Introduction of this paper, and the interpretation of the results was given in connection with the experimental data. It only remains, therefore, to bring together the main conclusions, and, without attempting to develop a theory to account for the relations, to summarize and present the findings which appear, at the present time, to be significant.

The hydrolyzing actions on ten different esters of extracts of whole rats of different ages, kept under uniform conditions for different lengths of time, changed in definite ways. The changes appeared to depend primarily upon the ages of the rats when killed. The changes in the actions on the individual esters, in the main, were decreases with time and decreases at different rates. The relative actions on the different esters of a given extract therefore changed and the pictures or types of the actions which mirror these relative actions consequently changed.

The results as presented may be grouped conveniently as follows: The extracts of the adult and old rats reached very nearly the same constant absolute values after standing 300 days or less. The pictures of these actions were similar to the pictures of embryo rat actions and the Flexner-Jobling rat carcinoma actions. Complete experimental results were given in this paper for two esters, glyceryl triacetate and methyl butyrate, for all the rats studied, and complete results on the ten esters for six rats of selected ages. The length of time of standing for an extract to reach the final embryonic or rat tumor type varied with the age of the rat when killed. For the youngest rats (up to 17 days of age), whose types were very nearly of the embryonic type to start with, the enzyme actions of the extracts either remained constant or increased for short periods of time. For young rats between the ages of 22 and 89 days, the change in type of the extracts to reach the embryonic or rat tumor picture was comparatively rapid; for adult rats, between the ages of 100 and 913 days, the change was much slower; while for the oldest rats, between the ages of 994 and 1110 days, the change was again more rapid.

In presenting these relations, the possible inaccuracies of the experimental method and the incompleteness of some of the data must be kept in mind. At the same time, in view of the large mass of data accumulated, only part of which can be communicated here, it is believed that the conclusions stated are amply justified by the experimental facts.

The significance of these changes in their bearing on the chemical nature of the enzymes may be discussed briefly.

Whether the hydrolyzing actions on a number of esters are due to one individual enzyme molecule, to a number of individual enzyme molecules, or to one active enzyme grouping in one or more complex molecules, or to several such active groupings, is a question which cannot be answered at present with the data available. Any statement would be essentially a personal opinion. The following discussion, even when such a personal opinion is presented would hold if one of the other views of the chemical nature of the active enzyme grouping were adopted.

In passing from the embryo to the adult rat, the ester-hydrolyzing enzymes increase in amount as well as in number. This is



shown by the larger actions on the individual esters, and by the changes in types or pictures which are due to the changes in the actions on the different esters.

In passing from the adult to the oldest rats, there is a decrease in the amounts of the actions, as well as a change in type. The change in type is in the direction of the embryo, so that it may be said, with the limitations above indicated, that both the amounts and the numbers of the enzymes decreased in old age.

The extracts of the whole rats on standing, in the course of time all showed the embryo type of action. If the type of action of the embryo originally is ascribed to one enzyme acting to different extents on different substrates, then the types of action of the adult rat might be ascribed either to one or more enzymes different from the former, or to one or more enzymes acting in addition to the enzyme acting in the embryo. On standing, the reversion of the type to the embryonic picture indicates that the latter view is probably the correct one, that the embryonic enzyme may be assumed to be present constantly, that it is supplemented by other enzymes in adult life, that in old age these latter enzymes begin to lose their actions, and that in the extracts these latter lose their actions more rapidly, leaving finally the embryonic type of enzyme which appears to possess the greatest stability. Furthermore, the new enzymes formed in the adult appear to possess different stabilities, depending upon the age of the rat. For the mature, adult rat, these enzymes are more stable than for the young rat or for the very old rat.

It is an interesting fact, but perhaps no more than a coincidence, that the embryo enzyme, as shown by its picture, is similar to several rat tumor enzymes. The enzymes of the adult rat or of the separate rat tissues appear, under certain conditions, to lose their individualities and to show only the enzyme characteristic of the rat embryo or tumor. It is possible, although only a suggestion at present, that the stabilities of the enzymes formed in the rats, different at different ages of the rats, are related to the resistance of the living rat to tumor formation at different ages.

Two possible points of view present themselves; the enzyme actions found are in themselves the important and perhaps predominating features of growth and of life processes, or the enzyme

actions studied are not directly responsible for the life processes, but are a characteristic property, important, and perhaps unique, as a chemical test for following certain properties, their stabilities and transformations, of the substances which are significant for life processes.

It must be remembered that enzymes are real substances with certain properties, although their chemical formulas are not known. This lack of knowledge, however, does not negative their existence as chemical substances which react with other substances to produce certain changes which may be followed and studied.

From the chemical point of view outlined thus briefly, the results presented have a broader bearing than if they are considered as empirical statements of certain properties of materials obtained from living matter. These relations will be developed further in subsequent papers.

The writers wish to thank Mr. Edmond White for his assistance in the experimental work.

#### SUMMARY.

The hydrolyzing actions on ten esters of extracts of whole rats were tested in the usual manner after standing under antiseptic conditions for different periods of time up to 23 months. Changes in absolute actions were found, which when considered as relative actions showed changes in the pictures or types of the actions. The extracts on standing all showed finally the embryo or Flexner-Jobling rat carcinoma type of action, but the rates at which the types changed were different depending upon the age of the rat when killed.

The object of this part of the investigation was stated in considerable detail, and some of the possible theoretical relations were outlined.



## STUDIES ON ENZYME ACTION.

### XLI. TIME CHANGES IN ESTER-HYDROLYZING ACTIONS OF EXTRACTS OF WHOLE MICE OF DIFFERENT AGES.

By K. GEORGE FALK AND HELEN MILLER NOYES.

(From the Harriman Research Laboratory, The Roosevelt Hospital,  
New York.)

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#### INTRODUCTION.

In the preceding paper,<sup>1</sup> the spontaneous changes in the ester-hydrolyzing actions of extracts of whole rats after different periods of time were presented. It was found that the pictures or types of action changed as the extracts were allowed to stand, that the rates of the changes depended on the ages of the rats when killed, and that ultimately all showed the type of action found for rat embryos and the Flexner-Jobling rat carcinoma. Since the types of action of mouse embryos (while similar to those of rat embryos) differed from those of the mouse carcinomas which have been studied,<sup>2</sup> it was the purpose of this phase of the investigation to determine whether the extracts of mice of different ages would on standing change so as to conform to the embryonic or to the mouse tumor type of action.

#### EXPERIMENTAL.

##### *Methods and Results.*

The methods used with mouse extracts were identical in all respects with those used with rat extracts and described in the preceding paper. The smaller sizes of the mice made it impossible to repeat the determinations as frequently and over such extended time periods as with the rats. The results obtained, however,

<sup>1</sup> Noyes, H. M., and Falk, K. G., *J. Biol. Chem.*, 1927, lxxii, 449.

<sup>2</sup> Falk, K. G., and Noyes, H. M., *J. Gen. Physiol.*, 1926-27, x, 359.

are sufficiently numerous to lead to definite conclusions. The experiments will not be given in detail. Six mice of different ages have been chosen as typical and the relative and absolute enzyme actions for these are shown in the figures.

The relative ester-hydrolyzing actions of extracts of the six mice of different ages immediately upon preparation and after standing are shown in the six charts of Fig. 1. For the youngest mouse (age 5 days), the type on standing changed to one approaching the mouse or rat embryo. For the older mice, the types or pictures after standing were clearly similar to those of the mouse tumors which have been studied. Although the results after standing in the different cases may not be strictly comparable because of the different time intervals, the changes in the types can readily be seen, especially with the older mice. Without going into detail it may be pointed out that the comparative actions, for example on methyl butyrate and benzyl acetate, and on benzyl acetate and ethyl acetate, lead to the conclusions stated. A study of other pairs of esters emphasizes these relations.

The absolute ester-hydrolyzing actions from which the relative actions shown in Fig. 1 were obtained, are given in Fig. 2. The actions decreased in every case even for the shortest periods of standing. The different rates of decreased actions for the various esters are clearly brought out in these charts. In general terms, the actions on the butyric esters decreased most rapidly, those on the acetic esters much less rapidly. The actions on benzyl acetate decreased perhaps least, especially with the five older mice. The magnitudes of the actions and of the changes also show that the relative actions may be considered to have a satisfactory degree of reliability to warrant the conclusions drawn.

Although the extracts did not stand sufficient lengths of time to make it possible to say that the pictures of the actions attained represented the final types, the results presented in the figures, the results with the extracts of sixteen additional mice which corroborated the results given, and the extracts which did stand longer periods of time, confirmed the conclusions stated in connection with Figs. 1 and 2.

In order to attempt to bring out the relations more clearly, the curves shown in Fig. 3 are presented. The relative enzyme actions on standing of extracts of a young mouse and of an old

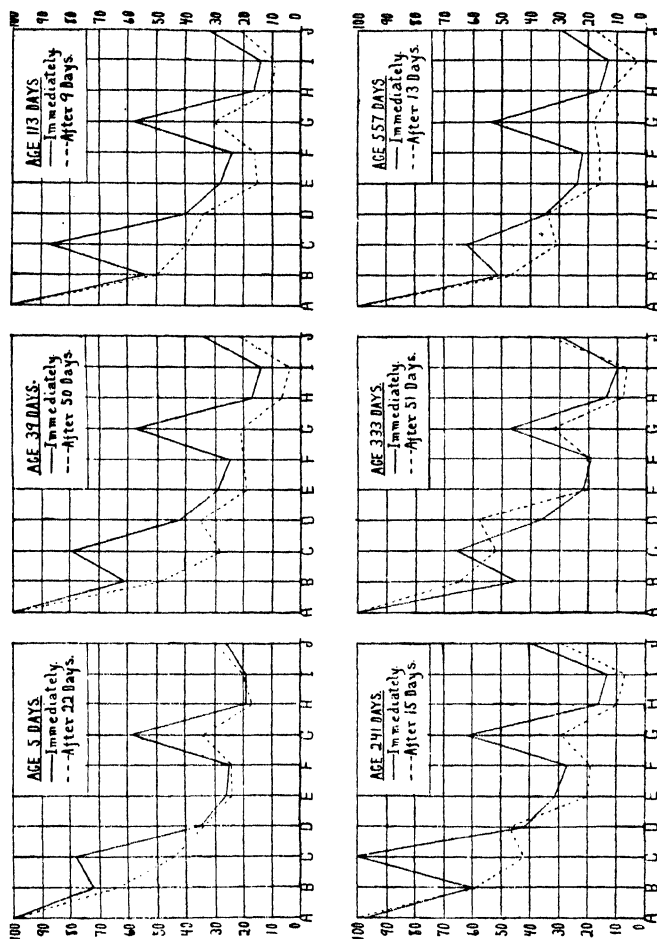


FIG. 1. Relative ester-hydrolyzing actions of whole mice of different ages. The esters are arranged in the usual order along the abscissa axis, the indicated positions being as follows: A phenyl acetate, B glyceryl triacetate, C methyl butyrate, D benzyl acetate, E ethyl acetate, F methyl acetate, G ethyl butyrate, H methyl benzoate, I ethyl benzoate, J isobutyl acetate. The curves for extracts of six mice are shown tested immediately and after standing the indicated numbers of days. The numbers of days standing were different for the different extracts, ranging from 9 to 51 days.

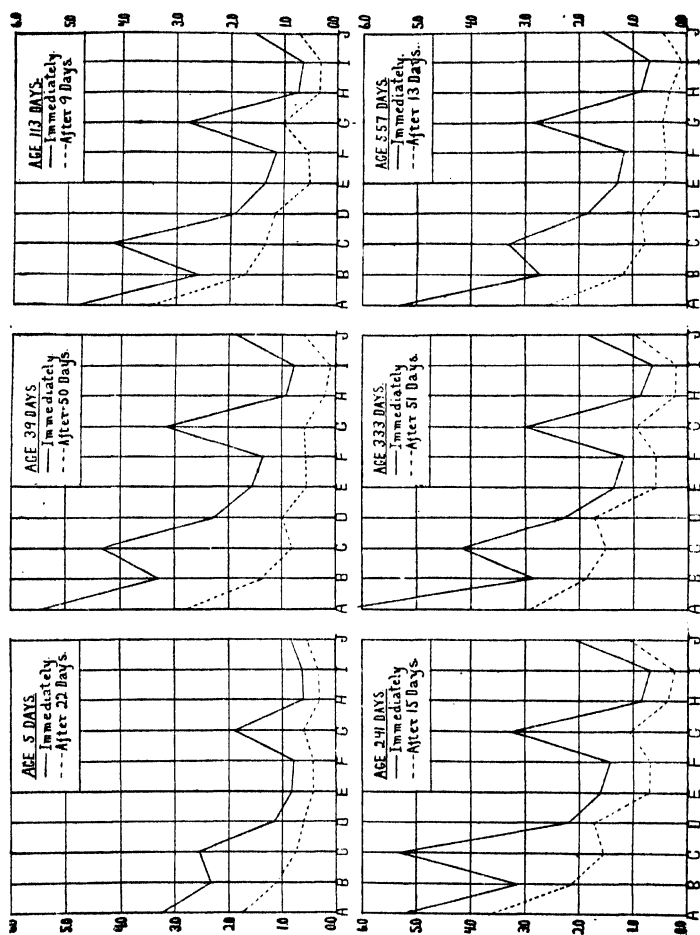


FIG. 2. Absolute actions in tenths of milli-equivalents of esters hydrolyzed by extracts of whole mice of different ages immediately and after standing the indicated numbers of days. These absolute actions refer to the same extracts for which the relative actions were shown in Fig. 1. The esters are arranged in the usual order along the abscissa axis, the indicated positions being the same as in Fig. 1.

mouse and of the average of three mouse carcinoma types and of a mouse embryo are given. Different mice from those shown in Figs. 1 and 2 were chosen. The detailed results for the mouse carcinomas and for the mouse embryo were presented elsewhere.<sup>3</sup>

The curves show clearly that the extracts of the older mouse on standing and the average of the extracts of the mouse carcinomas coincide very closely. The picture of the extract of the younger mouse on standing differed considerably from the picture of the

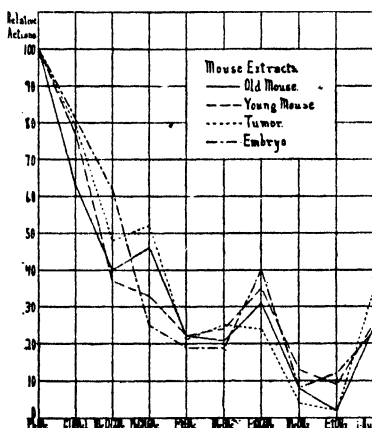


FIG. 3. Curves of relative ester-hydrolyzing actions of mouse tumors (average of Bashford 63 and Twort carcinomas and S 37 sarcoma) of mouse embryo (6 days before birth), and of two mouse extracts, one of a 4 day mouse after standing 42 days, and the other of a 466 day mouse after standing 50 days. The similarity of the curves of the tumor extract and older mouse extract on standing is evident. The younger mouse extract on standing differs somewhat from the tumor extract in the direction of the mouse embryo extract although it does not coincide with the latter.

tumor extract. It approached that of the mouse embryo and although not identical with it, may be said to be more like it than like the mouse tumor. The following comparisons of the actions on pairs of esters bring out these relations: the benzyl acetate: methyl acetate ratios are 1.38:1 for the young mouse; 1.32:1 for the mouse embryo; 2.19:1 for the old mouse, and

<sup>3</sup> Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1925, ix, 129.



2.08:1 for the mouse tumors; the phenyl acetate:benzyl acetate ratios are 3.03:1 for the young mouse, 4.00:1 for the mouse embryo, 2.17:1 for the old mouse, and 1.94:1 for the mouse tumor; etc. All the ratios of actions which might be chosen do not point as clearly to the same conclusions, but the trend is definite enough to make the similarity between the actions of the extracts of the older mouse and of the tumors quite certain, while the actions of the extract of the younger mouse differ apparently and approach the actions of the extract of the mouse embryo. At the same time, it may be recalled that the curve for the mouse embryo is not as definite as the curves for the rat embryos which have been determined. This may add to the uncertainty of some of the comparisons.

#### DISCUSSION.

The experimental data outlined and presented in part in this paper may be said to answer with a considerable degree of positiveness the problem set in the Introduction. The extracts of the young mice on standing tend in the direction of the embryo type of action; those of the adult mice become similar to the mouse tumor type of action. The results do not indicate the exact age at which this change in function between the young and old mice occurs, but as far as this can be approximated with the mice studied it appears to take place between the ages of 8 and 12 days.

The changes in the enzyme actions of the mouse extracts taken in connection with the changes in the enzyme actions of the rat extracts described in the preceding paper lead to some suggestive possibilities. With the adult animals, the rat extracts changed their types of action, becoming finally embryonic or tumor-like (Flexner-Jobling rat carcinoma or Jensen rat sarcoma) in type. As shown in previous papers, these rat tumor types were quite similar to the rat embryo types. It was impossible to decide with the rats alone whether the final actions belonged to the embryonic or to the tumor type if in fact such a distinction could be made. If it is permissible to carry over conclusions of the mouse experiments to the rats, and this is manifestly an assumption, it would be said that the final type reached by the rat extracts on standing may be considered to be

actions representative of the rat tumors rather than of the rat embryos. The spontaneous changes which occur in the substances present in adult rat and mouse extracts lead to the formation of substances giving the types of enzyme actions characteristic of certain tumors peculiar to the particular animal species. The question may therefore be asked whether the changes in the substances which lead to the same final types of enzyme actions are not only analogous but similar, and whether therefore the study of the chemical changes occurring in the extracts of the animal may not only parallel some of the abnormal changes in the living organism, but possibly, when sufficient data are available, also throw light on them.

#### SUMMARY.

The hydrolyzing actions on ten esters of extracts of whole mice were tested in the usual manner after standing under antiseptic conditions for different periods of time. The absolute actions decreased on standing and the types or pictures of the relative actions changed. The type of action found with the extracts of the adult mice on standing corresponded very closely to the types of the mouse carcinomas which have been studied and differed from the mouse embryo type of action. The extracts of the very young mice on standing did not change to the mouse tumor type of action but approached to some extent the mouse embryo type.



## STUDIES ON ENZYME ACTION.

### XLII. TIME CHANGES IN ESTER-HYDROLYZING ACTIONS OF EXTRACTS OF THE TWO TYPES OF UTERINE FIBROIDS AND OF UTERINE MUSCLE OF HUMAN ORIGIN.

By HELEN MILLER NOYES AND K. GEORGE FALK.

(From the Harriman Research Laboratory, The Roosevelt Hospital,  
New York.)

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#### INTRODUCTION.

It was shown in previous papers that human uterine fibroids could be grouped in two distinct classes by means of their ester-hydrolyzing actions.<sup>1</sup> The differences between these two classes, called Type I and Type II for convenience, were not reflected in the histological findings or physical properties of the tumors as far as could be told. The changes on standing in the ester-hydrolyzing actions of these fibroids as well as of uterine muscle will be presented in this paper.

#### EXPERIMENTAL.

##### *Methods and Results.*

The experimental methods were the same in all respects as those used in the work described in the preceding papers. They will, therefore, not be repeated here.

##### *Type I Fibroid Results.*

The curves of the relative ester-hydrolyzing actions of the Type I fibroids corresponded very closely to the curves of human bladder tumors, of certain embryonic human and animal tissues, and of the Flexner-Jobling rat carcinoma and the Jensen rat

<sup>1</sup> Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Cancer Research*, 1925, ix, 105. Falk, K. G., and Noyes, H. M., *J. Cancer Research*, 1926, x, 146.

sarcoma. This was shown in previous publications.<sup>1,2</sup> The absolute actions were comparatively small on all the esters except phenyl acetate and glyceryl triacetate. This is brought out in Table I, where ester-hydrolyzing actions in terms of tenths of milli-equivalents of esters hydrolyzed (or cc. of 0.1 N sodium hydroxide solution used in the titrations corrected for blanks) found with extracts of three fibroids, whose concentrations corresponded to 44.4 mg. of original material extracted per cc. of final solution tested, are given. These three series were chosen from thirty-seven such as representing the largest actions, the

TABLE I.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Three Type I Fibroid Extracts (Concentrations 44.4 Mg. of Material Extracted per Cc. of Solution Tested).*

Ester.	Experiment No.		
	R100A	R62	R78A
PhOAc.....	1.22	0.37	0.78
Gl(OAc) <sub>3</sub> .....	0.77	0.28	0.51
MeOCOPr.....	0.28	0.13	0.23
PhCH <sub>2</sub> OAc.....	0.20	0.02	0.14
EtOAc.....	0.12	0.00	0.10
MeOAc.....	0.23	0.10	0.17
EtOCOPr.....	0.29	0.08	0.14
MeOBz.....	0.00	0.00	0.00
EtOBz.....	0.00	0.00	0.00
i-BuOAc.....	0.18	0.02	0.19

smallest actions, and an intermediate more or less average set, found.

All of the ester-hydrolyzing actions of the Type I fibroid extracts decreased as the extracts were allowed to stand. Because of the small initial actions of the extracts which are shown in Table I, the actions after different time intervals will not be very significant because of the comparative magnitudes of the possible experimental errors. The detailed changes therefore will not be

<sup>2</sup> Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925-26, viii, 75.

given, but it may be stated that in the changes in actions which occurred, in no case was there any indication that the type or picture of the action had changed. Nine fibroids were studied in which the concentrations of the extracts were twice as large. Because of the larger amounts of materials required, it was not possible to study these extracts over extended time periods. The absolute actions of two of these Type I fibroid extracts, concentration 88.9 mg. of original material per cc. of extract tested, immediately upon preparation and after various times of standing are presented in Table II.

TABLE II.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Two Type I Fibroid Extracts (Concentrations 88.9 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

Ester.	R39				R36		
	0 days.	15 days.	33 days.	65 days.	0 days.	19 days.	32 days.
PhOAc.....	1.28	1.08	0.99	0.97	1.97	1.53	1.44
Gl(OAc) <sub>3</sub> .....	1.05	0.75	0.66	0.66	1.41	0.99	0.87
MeOCOPr.....	0.35	0.33	0.33	0.36	0.47	0.48	0.48
PhCH <sub>2</sub> OAc.....	0.31	0.19	0.22	0.23	0.49	0.33	0.25
EtOAc.....	0.22	0.16	0.16	0.19	0.33	0.25	0.21
MeOAc.....	0.34	0.25	0.20	0.24	0.44	0.35	0.33
EtOCOPr.....	0.15	0.16	0.13	0.16	0.24	0.27	0.23
MeOBz.....	0.03	0.07	0.05	0.11	0.18	0.23	0.18
EtOBz.....	0.00	0.02	0.03	0.05	0.01	0.06	0.04
i-BuOAc.....	0.37	0.21	0.19	0.27	0.54	0.30	0.26

Even with the more concentrated extracts, for which the results are given in Table II, the actions on the esters following the first two were small. These actions in general decreased as the extracts were allowed to stand. With the possible experimental errors being comparatively large, it is clear that the types of the actions did not change on standing. The calculation of the relative actions of the different extracts initially and after standing brings this out still more clearly, but the results already presented may be sufficient for the present purpose.

*Type II Fibroid Results.*

The Type II fibroid extracts behaved quite differently from the Type I fibroid extracts on standing. The relative actions are shown in Fig. 1 for the averages of forty-four extracts immediately upon their preparation and for the averages of a number of the extracts after five different time periods.

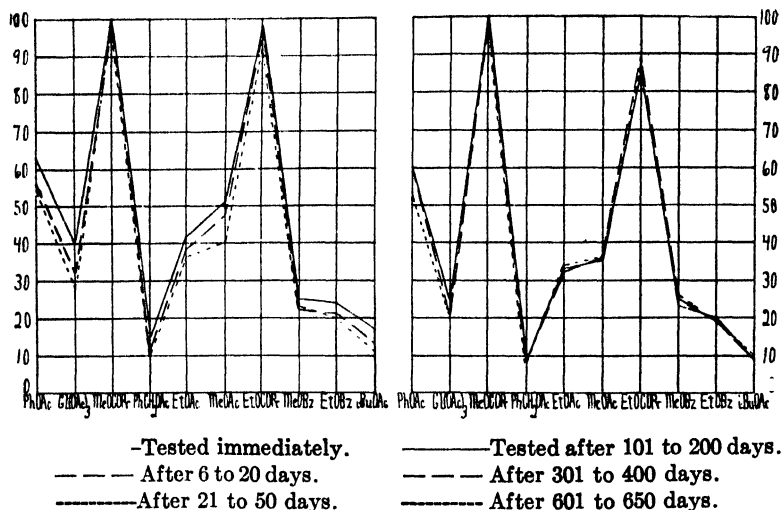


FIG. 1. Relative ester-hydrolyzing actions of Type II fibroid extracts immediately and after standing the indicated numbers of days. Average values are shown, forty-four extracts tested immediately, twenty-nine after standing between 6 and 20 days for the different extracts, twenty after standing 21 to 50 days, thirteen after standing 101 to 200 days, eleven after standing 301 to 400 days, and five after standing 601 to 650 days. The change in type is not great but still quite distinct. The pictures shown in the last three curves are practically the same, showing that the final type of action had been attained.

The curves shown in Fig. 1 indicate some real changes in the relative ester-hydrolyzing actions of the Type II fibroids on standing. The most striking changes are the relative decreases to about one-half of their original values of the actions on glycerol triacetate, benzyl acetate, and isobutyl acetate in comparison with the methyl butyrate actions which remained greatest throughout. The actions on phenyl acetate, ethyl acetate, and ethyl

TABLE III.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Five Type II Fibroid Extracts (Concentrations 44.4 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

Ester.	R74A. Days standing.						
	0	7	27	61	284	379	643
PhOAc.....	0.82	0.83	0.99	1.46	1.48	1.27	1.31
Gl(OAc) <sub>2</sub> .....	0.65	0.59	0.46	0.62	0.50	0.55	0.58
MeOCOPr.....	1.72	1.87	1.93	2.68	2.50	2.59	2.66
PhCH <sub>2</sub> OAc.....	0.36	0.25	0.12	0.21	0.25	0.18	0.18
EtOAc.....	0.69	0.74	0.67	0.99	0.80	0.92	0.96
MeOAc.....	0.93	1.02	0.80	1.04	0.81	1.05	1.04
EtOCOPr.....	1.74	1.92	1.87	2.45	2.09	2.45	2.40
MeOBz.....	0.46	0.40	0.40	0.55	0.59	0.72	0.69
EtOBz.....	0.49	0.43	0.35	0.51	0.50	0.48	0.57
i-BuOAc.....	0.39	0.34	0.17	0.27	0.27	0.23	0.29

	R91A. Days standing.					
	0	43	76	270	332	535
PhOAc.....	0.96	1.08	1.27	1.08	1.18	1.10
Gl(OAc) <sub>2</sub> .....	0.62	0.40	0.46	0.43	0.45	0.42
MeOCOPr.....	1.42	2.21	2.16	2.13	2.21	2.14
PhCH <sub>2</sub> OAc.....	0.23	0.10	0.25	0.12	0.12	0.15
EtOAc.....	0.62	0.61	0.73	0.70	0.67	0.74
MeOAc.....	0.71	0.69	0.76	0.75	0.78	0.76
EtOCOPr.....	1.40	1.91	1.70	1.91	1.95	1.98
MeOBz.....	0.44	0.44	0.55	0.58	0.49	0.59
EtOBz.....	0.39	0.39	0.44	0.40	0.40	0.38
i-BuOAc.....	0.29	0.12	0.19	0.15	0.16	0.17

	R98B. Days standing.				
	0	4	11	52	92
PhOAc.....	1.00	1.12	1.32	1.45	1.42
Gl(OAc) <sub>2</sub> .....	0.63	0.63	0.62	0.61	0.65
MeOCOPr.....	1.61	1.96	2.35	2.83	2.63
PhCH <sub>2</sub> OAc.....	0.26	0.25	0.23	0.19	0.21
EtOAc.....	0.68	0.84	0.88	1.02	0.99
MeOAc.....	0.78	0.92	1.00	1.10	1.09
EtOCOPr.....	1.63	1.91	2.27	2.69	2.45
MeOBz.....	0.50	0.48	0.65	0.64	0.74
EtOBz.....	0.41	0.44	0.48	0.45	0.46
i-BuOAc.....	0.29	0.28	0.29	0.17	0.28



TABLE III—*Concluded.*

Ester.	R103C. Days standing.				R75B. Days standing.		
	0	22	66	208	0	18	60
PhOAc.....	1.14	1.23	1.20	1.08	0.47	0.72	0.88
Gl(OAc) <sub>3</sub> .....	0.48	0.47	0.47	0.33	0.31	0.33	0.36
MeOCOPr.....	1.79	1.93	1.83	1.51	1.05	1.28	1.72
PhCH <sub>2</sub> OAc.....	0.16	0.12	0.12	0.12	0.00	0.06	0.11
EtOAc.....	0.67	0.63	0.51	0.43	0.31	0.43	0.52
MeOAc.....	0.77	0.68	0.58	0.47	0.50	0.56	0.54
EtOCOPr.....	1.65	1.75	1.54	1.29	1.06	1.22	1.52
MeOBz.....	0.38	0.48	0.42	0.38	0.13	0.23	0.27
EtOBz.....	0.32	0.38	0.36	0.32	0.16	0.28	0.30
i-BuOAc.....	0.16	0.16	0.14	0.16	0.05	0.07	0.06

butyrate decreased somewhat, those on methyl acetate to a greater extent. The type of curve evidently changed as the extracts were allowed to stand. Although it is difficult to state the exact length of time when the change in type was complete, on the basis of the curves of the average relative actions it may be said to have been between 3 and 4 weeks. The curves appear to have reached a definite type then, the changes thereafter being small and evidently accidental. Of course, individual fibroid extracts may show much greater or smaller times when the type has become fixed, but the average time is given on the basis of the results in Fig. 1.

A study of the absolute ester-hydrolyzing actions of the Type II fibroid extracts on standing shows relations entirely different from the Type I fibroid extracts. It was found that the actions on a number of the esters increased as the extracts were allowed to stand, reaching maximum values after different periods of time, and then in some instances decreasing again. Several of these experiments are given in Table III.

The results in Table III show the absolute actions of the five extracts (concentrations of 44.4 mg. of original tumors extracted per cc. of final solutions) after standing the indicated numbers of days. They may be considered to be more or less typical of the behaviors found. The following conclusions, based upon the behaviors of forty-four Type II fibroids, may be presented.

Increases in the enzyme actions were observed on certain

esters and not on others.<sup>3</sup> This indicates that the general conditions for the actions (such as a slightly more favorable pH, etc.) are not in themselves the cause of the increases as otherwise the actions would be expected to be increased on all the esters. The increases are specific to a considerable extent. In general, the actions on the butyrates increase most and those on glyceryl triacetate, benzyl acetate, and isobutyl acetate least. Increases amounting to over 50 per cent of the original actions were observed a number of times.

While increases in the enzyme actions were observed with a considerable number of the extracts, these increases did not occur uniformly or regularly. With some of the extracts, the actions remained constant over long time periods, with some there were decreases in actions, but with the majority the actions on a number of the esters increased, reached a maximum, and finally decreased again. While the rates of increases differed, it may be said that as a rule, the maximum actions were not reached until the extracts had stood about 50 days.

These extracts were allowed to stand at room temperatures whose extremes were about 10 and 30°, but for which the general run ranged between 15 and 25°. A number of extracts was kept at the incubator temperature (37-38°) as well as at the room temperature and the actions determined after different intervals of time. Table IV gives the results of two experiments for both temperatures, and the results of one experiment (R74A) at incubator temperature for which the results at room temperature were given in Table III. For convenience the room temperature is given as 20°, its general average.

The striking feature of the extracts kept at 38° is the way in which they retained their enzyme activity. The kind of change which the actions undergo is the same as that at the lower temperature, although the rate of the change is greater. Experiment R71 showed more rapid increases in actions at the higher temperature followed later by considerable decreases. This experiment is interesting in that at both temperatures the actions on all the esters increased or remained constant until at the higher tempera-

<sup>3</sup> Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Am. Chem. Soc.*, 1924, xlii, 1885.

TABLE IV.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Three Type II Fibroid Extracts (Concentrations 44.4 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time at Two Temperatures.*

Ester.	R71. Days standing at 20°.					
	Immediate tests.	9	24	63	148	645
PhOAc.....	1.16	1.13	1.35	1.50	1.66	1.40
Gl(OAc) <sub>3</sub> .....	0.79	0.56	0.53	0.63	0.68	0.53
MeOCOPr.....	1.81	2.08	1.94	2.17	2.24	2.47
PhCH <sub>2</sub> OAc.....	0.27	0.24	0.14	0.23	0.15	0.23
EtOAc.....	0.69	0.66	0.62	0.63	0.57	0.80
MeOAc.....	0.82	0.80	0.59	0.70	0.65	0.89
EtOCOPr.....	1.68	1.89	1.74	1.87	1.59	2.12
MeOBz.....	0.38	0.40	0.46	0.39	0.63	0.63
EtOBz.....	0.38	0.41	0.37	0.38	0.51	0.51
i-BuOAc.....	0.30	0.23	0.11	0.20	0.25	0.21

	Days standing at 38°.						
	1	2	3	4	6	13	42
PhOAc.....	1.12	1.05	1.11	1.14	1.14	0.89	0.99
Gl(OAc) <sub>3</sub> .....	0.67	0.61	0.61	0.62	0.69	0.51	0.32
MeOCOPr.....	1.99	2.20	2.30	2.30	2.40	2.24	1.38
PhCH <sub>2</sub> OAc.....	0.23	0.22	0.25	0.20	0.23	0.14	0.15
EtOAc.....	0.68	0.70	0.73	0.69	0.82	0.64	0.42
MeOAc.....	0.84	0.83	0.86	0.81	0.90	0.79	0.43
EtOCOPr.....	1.81	2.06	2.08	2.03	2.13	2.05	1.23
MeOBz.....	0.41	0.41	0.46	0.45	0.51	0.42	0.41
EtOBz.....	0.46	0.43	0.47	0.46	0.53	0.42	0.33
i-BuOAc.....	0.23	0.19	0.21	0.24	0.23	0.17	0.12

	R73. Days standing at 20°.				Days standing at 38°.			
	Immediate tests.	8	23	62	1	2	8	41
PhOAc.....	1.00	0.90	1.11	1.08	0.83	0.81	0.71	0.93
Gl(OAc) <sub>3</sub> .....	0.62	0.53	0.56	0.47	0.49	0.39	0.41	0.30
MeOCOPr.....	1.77	1.64	1.69	1.73	1.62	1.64	1.57	1.86
PhCH <sub>2</sub> OAc.....	0.26	0.15	0.17	0.12	0.18	0.16	0.16	0.12
EtOAc.....	0.88	0.82	0.81	0.84	0.78	0.79	0.73	0.61
MeOAc.....	1.05	0.94	0.86	0.98	0.89	0.90	0.89	0.59
EtOCOPr.....	1.62	1.49	1.56	1.51	1.49	1.43	1.51	1.66
MeOBz.....	0.34	0.27	0.36	0.34	0.30	0.24	0.27	0.41
EtOBz.....	0.48	0.27	0.31	0.34	0.31	0.26	0.33	0.32
i-BuOAc.....	0.30	0.23	0.21	0.18	0.21	0.14	0.18	0.12

TABLE IV—*Concluded.*

	R74A. Days standing at 38°.				
	Imme- diate tests.	1	2	13	78
PhOAc.....	0.82	0.76	0.86	0.80	0.86
Gl(OAc) <sub>3</sub> .....	0.65	0.51	0.54	0.52	0.44
MeOCOPr.....	1.72	1.81	2.02	2.47	2.44
PhCH <sub>2</sub> OAc.....	0.36	0.26	0.23	0.21	0.12
EtOAc.....	0.69	0.67	0.82	0.80	0.84
MeOAc.....	0.93	0.95	1.09	0.98	0.96
EtOCOPr.....	1.74	1.87	2.06	2.31	2.32
MeOBz.....	0.46	0.45	0.47	0.50	0.66
EtOBz.....	0.49	0.47	0.47	0.54	0.50
i-BuOAc.....	0.39	0.24	0.31	0.28	0.17

ture the large decreases, especially on the butyrates, were found. Experiment R73 gave results of practical constancy for all the actions at both temperatures. The variations noted in this experiment are within the errors of the various determinations. It is significant that even after 41 days at the higher temperatures the actions in general had not decreased. Experiment R74A showed increases at the higher temperature and no tendency toward decreases even after 78 days. The enzyme actions of this extract showed the same behavior at the two temperatures.

The stability of these enzyme actions, for the long times of standing at room temperature and at the incubator temperature, are particularly striking. It was shown in an earlier paper that the actions were completely destroyed if the extracts were boiled for 5 minutes. Heating the extracts for some days at 60° also destroyed the actions completely. No attempt was made to determine the exact stabilities of the enzyme actions with reference to temperature and time.

#### *Uterine Muscle Results.*

The changes on standing in the relative actions of the uterine muscle extracts are shown in Fig. 2. Not as many specimens were available as with the fibroids. The results are therefore collected and grouped arbitrarily for the different time periods.

The general course of the changes appears however to be fairly definite.

The change in type appeared to be complete after the first time interval, differing in this respect from the Type II fibroid where the change appeared to take place more slowly. The greatest differences in the relative actions with the uterine muscle extracts were the decreases on phenyl acetate and glyceryl tri-

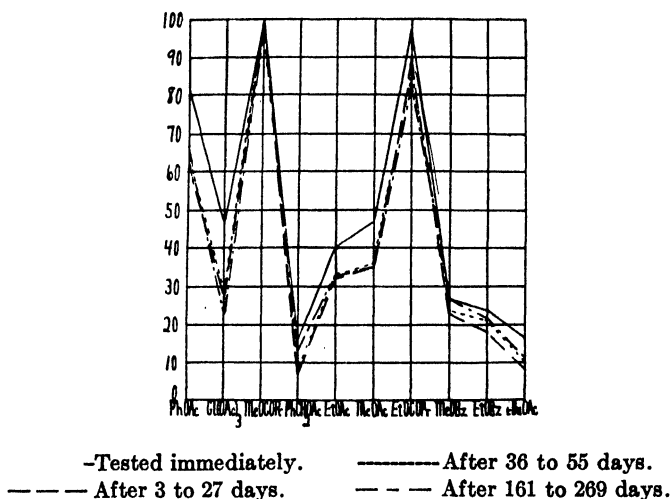


FIG. 2. Relative ester-hydrolyzing action of uterine muscle extracts immediately and after the indicated numbers of days. Average values are shown, eight extracts tested immediately, five after standing 3 to 27 days, seven after standing 36 to 55 days, and eight after standing 161 to 269 days. The change in type took place rapidly, the curves found immediately upon the preparation of the extracts being different from the other curves which coincided very closely with each other.

acetate, the decreased actions relative to the methyl butyrate actions on the remaining esters being smaller.

The absolute actions of four uterine muscle extracts, concentrations of 44.4 mg. of original material per cc. of extract as tested, are given in Table V, tested immediately and after standing the indicated numbers of days.

The uterine muscle extracts showed selective increases in the actions just as the Type II fibroid extracts did. Some of the

extracts showed no increases in any of the actions on standing, others again showed 50 per cent or larger increases. Just as with the Type II fibroids the greatest increases were observed on the butyrates. Here also, the fact that the increases were found

TABLE V.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Four Uterine Muscle Extracts (Concentrations 44.4 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

Ester.	R91B. Days standing.				R97F. Days standing.		
	0	43	176	269	0	52	253
PhOAc.....	1.47	1.71	1.86	1.63	1.07	0.97	0.85
Gl(OAc) <sub>3</sub> .....	0.88	0.69	0.54	0.46	0.61	0.49	0.32
MeOCOPr.....	1.99	3.07	2.85	2.72	1.12	1.33	1.12
PhCH <sub>2</sub> OAc.....	0.35	0.31	0.34	0.26	0.17	0.12	0.10
EtOAc.....	0.90	1.08	0.90	0.82	0.38	0.42	0.34
MeOAc.....	1.11	1.12	0.99	0.82	0.46	0.47	0.38
EtOCOPr.....	2.00	2.51	2.31	2.22	1.07	1.17	0.94
MeOBz.....	0.70	0.78	0.76	0.70	0.24	0.36	0.32
EtOBz.....	0.62	0.71	0.60	0.56	0.22	0.36	0.29
i-BuOAc.....	0.36	0.35	0.33	0.26	0.20	0.14	0.14

	R98D. Days standing.			R101B. Days standing.		
	0	4	11	0	40	230
PhOAc.....	1.23	1.33	1.36	1.53	1.75	1.90
Gl(OAc) <sub>3</sub> .....	0.70	0.66	0.52	0.84	0.79	0.65
MeOCOPr.....	1.59	2.14	2.33	2.10	3.08	3.14
PhCH <sub>2</sub> OAc.....	0.22	0.12	0.06	0.31	0.25	0.30
EtOAc.....	0.56	0.75	0.65	0.93	1.13	1.09
MeOAc.....	0.72	0.82	0.76	1.06	1.16	1.14
EtOCOPr.....	1.58	1.98	2.08	2.11	2.72	2.56
MeOBz.....	0.50	0.52	0.45	0.63	0.74	0.76
EtOBz.....	0.42	0.40	0.36	0.54	0.65	0.62
i-BuOAc.....	0.25	0.21	0.11	0.38	0.33	0.32

on certain esters while the actions on other esters remained constant or decreased, shows that the increases were not caused by a general change in the conditions of testing, but rather by changes in the compositions of the extracts which may be said to be specific



## DISCUSSION OF RESULTS.

The differences in the relative ester-hydrolyzing actions of the Type I and Type II fibroids were emphasized in former papers together with the fact that these differences were not reflected in the histological findings or other properties of these tumors.<sup>1</sup> The changes on standing of these fibroid extracts also showed marked differences, the enzyme actions of the Type I fibroid extracts diminishing with time while the pictures of the actions as far as could be told remained constant, while with the Type II fibroid extracts the pictures changed somewhat with time while the absolute actions on some of the esters increased markedly.

With the Type II fibroids and the uterine muscle, the pictures of the actions differed to some extent with the freshly prepared extracts, but after standing they coincided very closely. The absolute actions showed similar increases or constancies for the two sets of materials. The similarity of the ester-hydrolyzing actions of the Type II fibroids and the uterine muscles, especially marked after extracts have been allowed to stand, and the difference of these from the Type I fibroids are quite striking.

The increases in some of the ester-hydrolyzing actions of the Type II fibroid and uterine muscle extracts have been explained at various times for similar phenomena to be due to the removal of inhibiting substances. Whether this statement is accepted or whether the increases are assumed to be due to decompositions or other changes in substances present with the formation of new enzyme materials, is at present of little significance, although such statements or assumptions may fill a psychological need. Experimental evidence is lacking to answer the question of the mechanism of the increases in the enzyme actions. It is planned to study this phase of the problem further in the near future. The fact that the increased actions are selective or specific, that they are found with certain esters and not with others, may be taken to indicate that there is more than one enzyme acting upon the different esters.

The stability of some of the Type II fibroid ester-hydrolyzing actions is quite striking. These actions showed no decrease even after 22 months at room temperatures. The stability was not studied for as long time periods at 38°, but for the lengths of



time followed, similar stabilities were found. Evidently the rate of loss of different enzyme actions or of the same enzyme action obtained from different sources is a relative matter. None of the enzyme actions of the extracts here studied was heat-stable ( $100^{\circ}$ ); on the other hand, some of them showed unexpected and remarkable stability at room temperatures and even at  $38^{\circ}$ .

One additional matter may be mentioned. The uterine muscle specimens which were studied were obtained as a result of operations in which they were removed because of the presence of fibroid masses. It is therefore a question whether these uterine muscles are to be considered as normal tissues. Apparently they were normal, but because of the presence in them of the various fibroids, it would be too much of an assumption to accept them as such. The results herewith presented must therefore be accepted with this limitation of the significance of the behavior found with the uterine muscle extracts.

#### SUMMARY.

The hydrolyzing actions on ten esters of extracts of human uterine fibroids (Types I and II) and human uterine muscle after different lengths of time of standing were studied in the manner described in the preceding papers. For Type I fibroids, similar to the Flexner-Jobling rat carcinoma and certain embryo tissues and rat and mouse embryos, the absolute actions, comparatively small initially, decreased on standing, while the picture did not change. For Type II fibroids, the type changed to a certain extent, the change being complete on the average in about 7 weeks, while the absolute actions in a number of cases increased on certain of the esters and decreased on others. The increases on the butyrates at times amounted to over 50 per cent. For the uterine muscle, the type of action changed to a certain extent, the change being complete in a comparatively short time (less than 4 weeks at most), the final type being very similar to the final picture of the Type II fibroid actions, while the absolute actions showed selective increases on certain of the esters similar to the increases observed with the Type II fibroid extracts.

## STUDIES ON ENZYME ACTION.

### XLIII. TIME CHANGES IN ESTER-HYDROLYZING ACTIONS OF EXTRACTS OF SOME RABBIT TISSUES.

BY K. GEORGE FALK AND HELEN MILLER NOYES.

(*From the Harriman Research Laboratory, The Roosevelt Hospital,  
New York.*)

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#### INTRODUCTION.

The ester-hydrolyzing actions of a number of tissues of rabbits whose ages ranged from 8 days before birth to 2162 days were presented in an earlier communication.<sup>1</sup> In this paper some of the results obtained with extracts of leg muscle, kidney, lung, and liver of rabbits of different ages, on standing at room temperature will be given. The methods used were identical in all particulars with those described in the preceding papers.

#### EXPERIMENTAL RESULTS.

Results obtained with the rabbit liver, lung, kidney, and leg muscle, will be presented here. In the first place, the changes in the relative actions of extracts of these four tissues immediately upon preparation and after standing different lengths of time are shown in Fig. 1. The tissues were taken from adult rabbits. The concentrations refer to 44.4 mg. of original material extracted per cc. of solution tested for the leg muscle; 17.8 mg. for the other three tissues. The times of standing were different for the four tissues, comparatively short for leg muscle, kidney, and lung, because of the small amounts of the materials, and much longer for the liver.

The important fact to be emphasized with the curves shown in Fig. 1, is that the change in type as the extracts of the different

<sup>1</sup> Noyes, H. M., Falk, K. G., and Baumann, E. J., *J. Gen. Physiol.*, 1925-26, ix, 651.

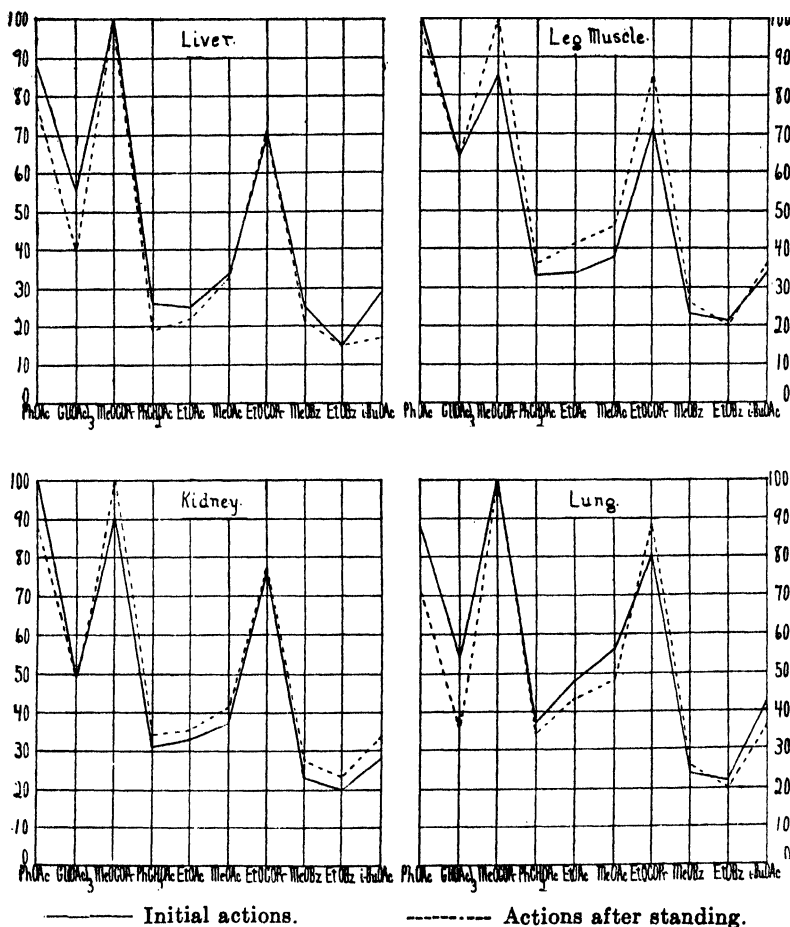
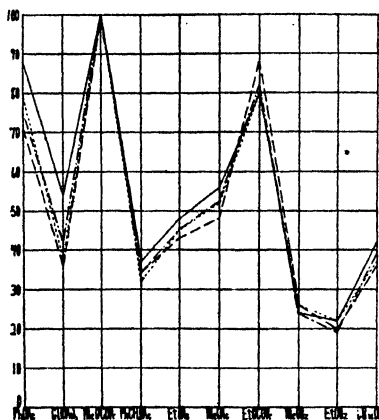


FIG. 1. Average relative ester-hydrolyzing actions of extracts of the liver, leg muscle, kidney, and lung of the adult rabbit immediately upon preparation and after standing 293 to 321, 16, 19 to 32, and 13 to 26 days, respectively. The changes in the four charts as shown by comparison of the solid lines and short dashes in each case showed that the relative ester-hydrolyzing actions of the extracts of the four tissues tended in the same direction, and away from the embryonic pictures of the actions.

tissues were allowed to stand was in every case in the same direction, the main features being the striking decreases in the actions on phenyl acetate and glyceryl triacetate relative to the methyl

butyrate and ethyl butyrate. These changes are in the same direction as the changes on standing observed with the Type II human uterine fibroid and human uterine muscle extracts which were described in the preceding paper. There is no indication whatsoever of a change in type in the direction of the embryonic picture described for whole rat embryos,<sup>2</sup> whole mouse embryos,<sup>3</sup>



———— Initial actions.                      - - - - - Actions after 119 to 180 days.  
 - - - - - Actions after 5 to 9 days.              - . - . - Actions after 293 to 321 days.

FIG. 2. Relative ester-hydrolyzing actions of extracts of livers of the adult rabbit immediately and after standing the indicated lengths of time. The curves refer to averages of the relative actions, nine sets of results for the immediate actions for rabbits between the ages of 180 and 2162 days, three sets after standing 5 to 9 days for rabbits 330 to 2162 days old, eight sets after standing 119 to 180 days for rabbits 180 to 2162 days old, and five sets after standing 293 to 321 days for rabbits 330 to 2162 days old. The change to a final type of action appears to be fairly rapid with these liver extracts. While the differences in the curves are not great, they are sufficient to show a real change in type.

and a number of tissues from embryo rabbits.<sup>1</sup> The rabbit leg muscle, kidney, and lung extracts were allowed to stand comparatively short times, but even in these short times the changes in

<sup>2</sup> Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925-26, viii, 75.

<sup>3</sup> Falk, K. G., and Noyes, H. M., *J. Gen. Physiol.*, 1926-27, x, 359.

relative actions and consequently in types, were clear cut. On the other hand, it cannot be said that these extracts had reached their final pictures. • The rabbit liver extracts, for which the curve for a long period of standing is given, have been studied more exhaustively in this connection.

In Fig. 2 are presented the curves of the relative actions of extracts of livers of adult rabbits of different ages immediately upon preparation, and after standing the stated lengths of time. The concentrations of these extracts correspond to 17.8 mg. of original material per cc. of solution tested. The curves represent averages of a number of series of determinations in each case, grouped according to the lengths of times of standing of the various extracts.

The results given in Fig. 2 show that the change in type of the liver extracts occurred quite rapidly, that the principal change had occurred within a week, and that further changes were of much less magnitude and perhaps secondary in character. This comparatively rapid change in type is more like that observed with human uterine muscle than that observed with human Type II uterine fibroid described in the preceding paper, the directions of the changes being similar however for these three (rabbit liver, human uterine muscle, human Type II uterine fibroid).

It was shown in an earlier paper that the pictures of the relative ester-hydrolyzing actions of most of the rabbit tissues studied were different for the embryo and very young rabbits in comparison with the adult rabbit. The rabbit liver, however, was found to be exceptional in this respect. The livers of the rabbit embryos showed types of action similar to those of the adult rabbits; for approximately 3 weeks after the birth of the rabbits, the types of actions of the livers reverted to a certain extent toward the embryonic type (as found with other tissues of rabbit embryos, and rat and mouse embryos), again becoming adult in type thereafter. The average relative actions of extracts of the livers of the very young rabbits and of the three oldest rabbits immediately upon preparation and after standing, are of interest therefore, and are shown in Fig. 3.

The first chart in Fig. 3 shows the average curve of the relative ester-hydrolyzing actions of extracts of livers of very young rabbits (age  $\frac{1}{2}$  to 28 days), immediately upon preparation, which

may be said to be between the curves of the adult rabbit liver and of other embryo rabbit tissues, and the average curve of the extracts after standing between 22 and 35 days. The type of the actions had changed definitely in the same direction as that shown for the adult rabbit liver and in a direction distinctly different

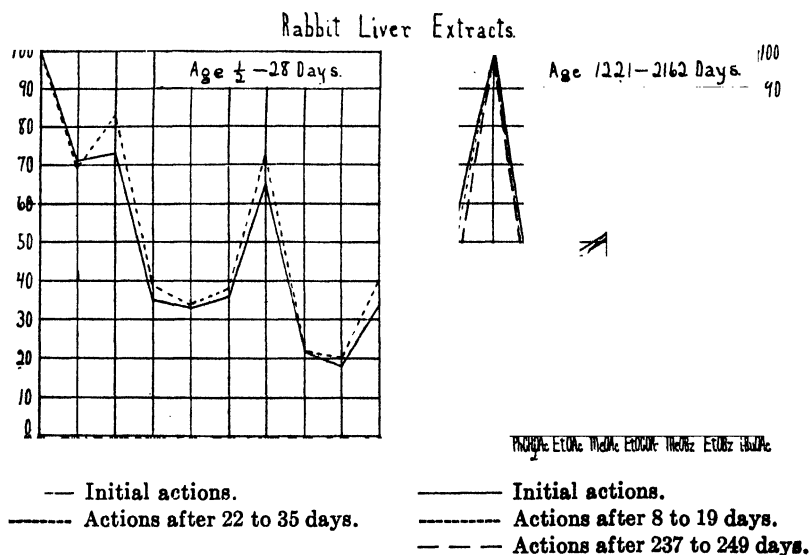


FIG. 3. Relative ester-hydrolyzing actions of extracts of livers of very young and old rabbits immediately and after standing the indicated numbers of days. Averages are shown; for the young rabbits the curves include nine extracts of livers of rabbits whose ages ranged from  $\frac{1}{2}$  to 28 days for the immediate actions, and seven extracts for the actions on standing, while the curves for the livers of the old rabbits include three extracts, the ages of the rabbits being between 1221 and 2162 days for the three curves. The trend of the curves of the relative actions was the same in both charts, away from the embryonic picture, although the curves of the initial actions were quite different in the two sets.

from that generally called embryonic. The second chart shows the three curves of the liver extracts of the oldest rabbits studied, the change in type from that found immediately to that found in a short period of standing (8 to 19 days), and the additional change for the long period (237 to 249 days) being indicated. This second chart brings out for the oldest rabbit livers the changes

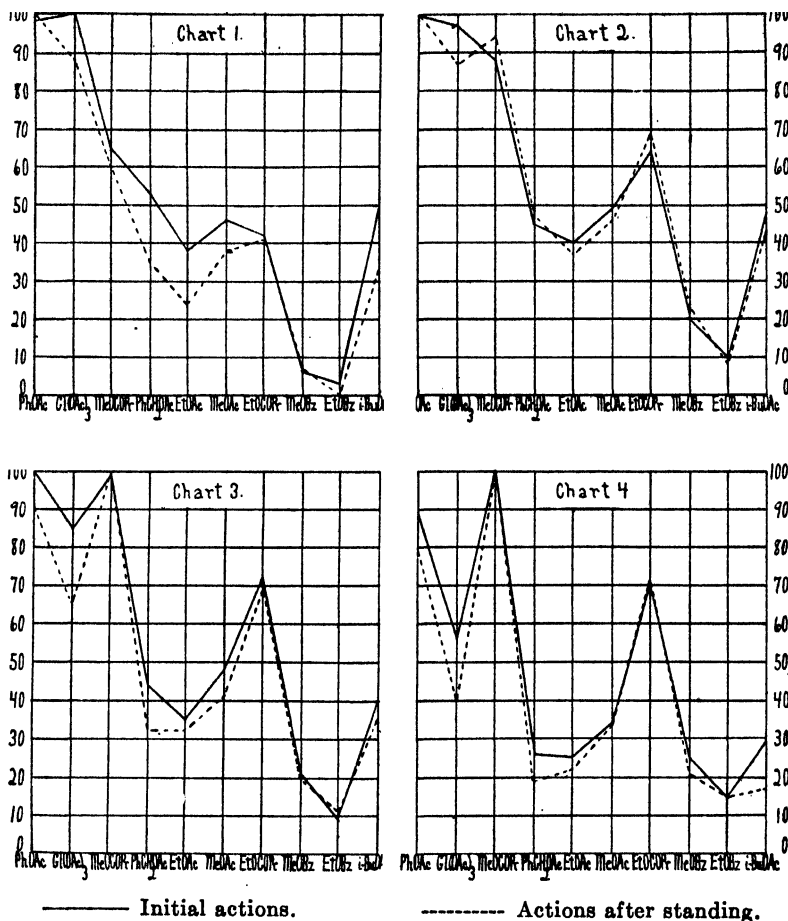


FIG. 4. Relative ester-hydrolyzing actions of extracts of leg muscle of rabbits of different ages immediately and after standing. The four charts refer to rabbits of four different ages, the first three to averages of two ages and times of standing for each, the last to one extract and one time of standing as follows: Chart 1, rabbit ages 21 and 35 days, times of standing 22 and 9 days; Chart 2, ages 45 and 67 days, standing 16 and 18 days; Chart 3, ages 102 and 136 days, standing 14 and 13 days; Chart 4, age 195 days, standing 16 days. The changes in the types of actions on standing are clear; reversion in the direction of the embryonic type for the youngest (Chart 1), clearly in an opposite direction for the oldest studied (Chart 4), and tending in the same direction as for the oldest, but not as distinctly for the intermediate ages (Charts 2 and 3).

shown in Fig. 2 for a large number of adult rabbits of wider age limits. Fig. 3 shows that the changes in type for liver extracts of the very young and the very old rabbits are of the same character.

Some results obtained with the rabbit leg muscle extracts are of interest in this connection. It was found previously that the ester-hydrolyzing actions of the leg muscle extracts obtained from very young rabbits approached the embryonic type while for the older rabbits an adult type was found. Four of these series of actions (three of them averages of extracts of leg muscles obtained from rabbits not differing much in age, the fourth being the results obtained with a single extract) are shown in Fig. 4, the solid lines representing the relative actions of the extracts immediately, the dotted lines the relative actions of the extracts after standing on the average about 2 weeks. The concentrations of the extracts corresponded to 44.4 mg. of original material per cc. of mixture tested.

There is a marked difference in the changes in relative actions as shown by the curves in Fig. 4, depending upon the ages of the rabbits when killed. The first chart, for the leg muscles of the youngest rabbits, showed a change in type to, or possibly a continuation of, the embryonic type. The last chart for the leg muscle of the 6 months old rabbit, showed a clear retention of the adult type of action. The other charts showed intermediate changes for the intermediate ages for these comparatively short lengths of time of standing. In considering these relative actions, it should be pointed out that the absolute actions, while not large, were sufficiently so for the determination of the course of the changes, and that only a limited number of extracts was studied in this way and over short time periods. Even with these limitations, the difference between the leg muscle extracts of the very young and the older rabbits is clear, the former tending to revert to the embryonic type of action on standing, the latter changing in an entirely different manner to a type which may perhaps be said to be characteristic of the changes observed with certain other adult tissues.

Experiments with lung and kidney extracts from rabbits of widely different ages were not carried out. The curves for these tissues shown in Fig. 1 refer only to materials obtained from adult animals.



Definite conclusions were drawn from the curves of the relative ester-hydrolyzing actions of the extracts of rabbit tissues im-

TABLE I.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Extracts of Livers of Three Rabbits of Different Ages (Concentrations 17.8 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

Ester.	E22C. Age 6½ days.		E13F. Age 195 days.				
	Days standing.		Days standing.				
	0	35	0	16	64	119	267
PhOAc . . . . .	3.01	1.48	5.90	5.22	4.51	4.38	4.04
Gl(OAc) <sub>3</sub> . . . . .	1.74	0.92	3.50	2.90	2.58	2.25	1.79
MeOCOPr . . . . .	1.74	1.18	6.38	7.29	5.76	5.65	5.14
PhCH <sub>2</sub> OAc . . . . .	0.92	0.52	2.27	2.06	1.84	1.67	1.54
EtOAc . . . . .	0.83	0.50	3.14	2.86	2.75	2.30	2.03
MeOAc . . . . .	0.81	0.60	3.58	3.14	2.66	2.66	2.38
EtOCOPr . . . . .	1.54	0.88	5.27	5.78	5.19	4.60	4.02
MeOBz . . . . .	0.62	0.38	1.39	1.53	1.42	1.31	1.31
EtOBz . . . . .	0.46	0.32	1.24	1.39	1.20	1.11	0.94
i-BuOAc . . . . .	0.82	0.52	2.69	2.40	1.99	1.83	1.71
E25K. Age 842 days.							
Days standing.							
	0	26	173	256	304	507	564
PhOAc . . . . .	3.61	2.72	3.13	2.53	2.60	2.41	2.30
Gl(OAc) <sub>3</sub> . . . . .	2.17	1.65	1.54	1.38	1.49	1.37	1.33
MeOCOPr . . . . .	3.90	3.42	3.67	3.20	3.19	3.09	3.09
PhCH <sub>2</sub> OAc . . . . .	1.40	1.20	1.32	1.18	1.23	1.20	1.09
EtOAc . . . . .	1.55	1.57	1.46	1.24	1.38	1.28	1.28
MeOAc . . . . .	1.80	1.68	1.85	1.51	1.45	1.40	1.49
EtOCOPr . . . . .	2.88	2.55	2.97	2.64	3.08	3.05	2.58
MeOBz . . . . .	0.95	0.80	0.98	0.81	0.91	0.88	0.85
EtOBz . . . . .	0.88	0.73	0.79	0.71	0.81	0.76	0.78
i-BuOAc . . . . .	1.64	1.30	1.40	1.20	1.39	1.28	1.22

mediately upon preparation and after standing. Some of the absolute actions which served for the calculation of the relative actions will next be presented. Three series, obtained with liver

extracts, are given in Table I, the first for the liver of a 6½ day rabbit, the second for the liver of a 195 day rabbit, and the third for an 842 day rabbit.

The liver extract results shown in Table I were chosen as typical

TABLE II.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Extracts of Leg Muscles of Seven Rabbits of Different Ages (Concentrations 44.4 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

No. of experiment.	E17A	E19B	E18A	E21E	E12R	E20B	E13C
Age of rabbit, days.	21	35	45	67	102	135	195
<b>Immediate actions.</b>							
PhOAc . . . . .	1.10	1.10	1.04	1.35	1.03	1.45	1.37
Gl(OAc) <sub>3</sub> . . . . .	1.11	1.03	1.08	1.21	0.89	1.22	0.86
MeOCOPr . . . . .	0.62	0.77	1.04	1.02	0.99	1.50	1.54
PhCH <sub>2</sub> OAc . . . . .	0.54	0.58	0.50	0.57	0.47	0.63	0.40
EtOAc . . . . .	0.38	0.42	0.46	0.47	0.38	0.49	0.39
MeOAc . . . . .	0.46	0.52	0.58	0.57	0.48	0.72	0.53
EtOCOPr . . . . .	0.31	0.58	0.78	0.73	0.71	1.10	1.08
MeOBz . . . . .	0.00	0.12	0.21	0.27	0.15	0.40	0.38
EtOBz . . . . .	0.00	0.06	0.12	0.11	0.08	0.14	0.23
i-BuOAc . . . . .	0.49	0.56	0.54	0.59	0.47	0.51	0.44
<b>Actions after, days.</b>							
	22	9	16	18	14	13	16
PhOAc . . . . .	1.00	0.85	1.02	1.03	0.90	1.37	1.19
Gl(OAc) <sub>3</sub> . . . . .	0.85	0.77	0.89	0.90	0.66	0.96	0.60
MeOCOPr . . . . .	0.55	0.55	1.02	0.90	0.96	1.58	1.51
PhCH <sub>2</sub> OAc . . . . .	0.29	0.34	0.48	0.47	0.31	0.50	0.29
EtOAc . . . . .	0.21	0.22	0.39	0.37	0.31	0.49	0.33
MeOAc . . . . .	0.37	0.32	0.50	0.44	0.38	0.65	0.50
EtOCOPr . . . . .	0.33	0.42	0.75	0.66	0.63	1.10	1.07
MeOBz . . . . .	0.04	0.08	0.24	0.23	0.13	0.39	0.31
EtOBz . . . . .	0.00	0.00	0.10	0.05	0.10	0.18	0.22
i-BuOAc . . . . .	0.24	0.36	0.43	0.45	0.38	0.48	0.26

examples. The important feature to be noted is that the enzyme actions were large so that the calculated relative actions may be viewed with a considerable degree of confidence. While there were decreases in the actions on standing in most cases, the actions on the whole remained surprisingly large, especially for the livers

of the adult rabbits, even after long periods of time. A certain amount of irregularity was apparent with the actions at successive tests. This is undoubtedly due to slightly changed conditions of testing the actions, such as failure to have the solution at exactly the same hydrogen ion concentration each time, etc. The course of the changes is, however, apparent. At times increased actions, especially on the butyrates (Experiment E13F for example), were observed, but there appeared to be no regularity, either of materials or of conditions, to account for, or to coordinate, these increases.

TABLE III.

*Absolute Actions in Tenths of Milli-Equivalents of Esters Hydrolyzed by Extracts of Kidneys of Two Rabbits of Different Ages (Concentrations 17.8 Mg. of Material Extracted per Cc. of Solution Tested) after Standing the Indicated Periods of Time.*

Ester.	E20K. Age 136 days.		E24D. Age 563 days.		
	Days standing.		Days standing.		
	0	13	0	28	176
PhOAc.....	3.75	2.89	2.73	2.10	1.96
Gl(OAc) <sub>3</sub> .....	3.20	2.12	1.89	1.36	0.94
MeOCOPr.....	2.89	3.35	2.30	2.08	1.78
PhCH <sub>2</sub> OAc.....	1.56	1.23	0.95	0.78	0.66
EtOAc.....	1.45	1.31	1.00	0.87	0.74
MeOAc.....	1.58	1.58	1.14	1.00	0.86
EtOCOPr.....	2.57	2.89	2.01	1.76	1.62
MeOBz.....	0.89	0.94	0.67	0.52	0.62
EtOBz.....	0.70	0.70	0.67	0.50	0.44
i-BuOAc....	1.51	1.19	1.01	0.79	

Since the leg muscle extracts showed differences in types of action upon immediate testing depending upon the age of the rabbit, the absolute actions for the seven experiments which were used for the calculation of the relative actions are given in Table II. The upper half of the table includes the immediate actions, the lower half the actions of the same extracts after standing the indicated numbers of days.

While the leg muscle extracts showed smaller actions than the liver extracts even in  $2\frac{1}{2}$  times greater concentrations, the changes on standing were sufficiently large to make their trend reasonably

certain. The actions of the extracts on the different esters on standing either decreased or remained constant. It is interesting to note that the largest decreases were found on glyceryl triacetate, while the decreases with the butyrates were practically zero.

The absolute ester-hydrolyzing actions found with two series of experiments with rabbit kidney extracts are given in Table III.

The results shown in Table III were large enough to warrant considerable reliance being placed on the curves of relative actions. These two series were quite typical of the behavior of the kidney extracts on standing. The decreases in the actions were not large. At times, selective increases, as shown by the butyrates in Experiment E20K, were found. While such increases were more or less isolated in the various experiments, they served to emphasize the specific characters of changes in the actions on the different esters as the extracts were allowed to stand. This specificity was shown by the different rates of decrease, not proportional to each other, of the actions on the different esters, which then, in turn, resulted in different curves for the relative actions as shown in Fig. 1.

The absolute actions of the lung extracts will not be given in detail since they were of the same order of magnitude as the kidney extracts, and the changes on standing, while naturally differing in various particulars, were of the same nature. Decreases were the general rule, occasional increases were found, the rates of change being different for the different esters, resulting in the changed type of curve shown in Fig. 1.

#### DISCUSSION.

Instead of limiting the discussion to the results presented in this paper, an attempt will be made to summarize the work presented in this series of four papers, and to indicate some of the points which have arisen.

It has long been known that tissues separated from living organisms undergo changes in various ways including changes in enzyme actions. It is these autolytic changes which have been studied here, but in place of following the changes in the tissues themselves, they have been followed in the extracts of these tissues, because such extracts can be more readily handled quantitatively. The

property of the tissues which was studied in this general investigation involved their ester-hydrolyzing actions under fixed conditions. These enzyme actions were chosen for reasons which were presented in detail elsewhere. The magnitudes of the enzyme actions were not the only property which was used in the study of various tissues, but a more significant relation was found in the comparison of the relative actions on the different esters. A convenient method of comparison which involved plotting the curves of the relative actions was developed. Tissues of various animals, both embryonic and adult, and tumors from various sources were found to show characteristic properties by this method of experimenting and treatment of the results. In following the autolytic changes of extracts of tissues and tumors, it was primarily this property of relative ester-hydrolyzing actions, secondarily the absolute ester-hydrolyzing actions, which were studied.

In determining these enzyme actions of the various tissue and tumor extracts, the conditions of testing were kept as uniform as possible. Where a change in hydrogen ion concentration of the extract had occurred, the latter was brought back to correspond to pH 7.0 as nearly as possible.

The question of specific changes in actions on certain esters, either increases or decreases, is of interest. For instance, the actions on certain esters were found to decrease much more rapidly than on others (the decrease in the butyrate actions for example with whole rat extracts), or the actions on certain esters increased markedly while those on other esters remained practically constant, or even decreased (for example, the increase in the butyrate actions with human Type II uterine fibroid extracts). These changes must be ascribed to the changes, which may be termed autolytic, in certain substances responsible for the enzyme actions in the extracts. Because of these changes, the relative actions or pictures of the actions of the extracts in question change, and it is such changes in pictures of enzyme actions which showed relationships analogous at times to certain physiological and pathological processes, and at other times raised pertinent questions.

The change in type of action of the whole rat extracts depended upon the age of the rat when killed. For the embryo and very youngest rats the type did not change on standing but remained similar to that of the Flexner-Jobling rat carcinoma, the Jensen

rat sarcoma, and certain embryo rabbit tissues. For the young rats the change to the above embryo, etc., type was fairly rapid as the extracts were allowed to stand, for the adult rats the changes were slower, and for the oldest rats the changes were more rapid again. The changes on standing all consisted of decreased actions, at different rates, for the different esters so that the types of the actions changed. The final type in every case was practically the same as that of the embryo rat and of a number of tissues of embryo rabbits. It was therefore called the embryo picture in earlier papers. Unfortunately it is the same type of picture of enzyme actions given by a number of tumors such as the Flexner-Jobling rat carcinoma, the Jensen rat sarcoma, human bladder tumors, human Type I uterine fibroids, and other human tumors. It was therefore impossible to decide whether the change in the relative enzyme actions of the whole rat extracts on standing meant a reversion to the embryo type or a change to the tumor type. An indication that the change is really one to the tumor type was found in the study of the whole mouse extracts, where the mouse embryo showed the embryo picture, but a number of mouse tumors showed characteristically different pictures. On standing the adult mouse extracts clearly changed to the mouse tumor type distinctly different from the type of the mouse and other embryos.

A study of separate rat and mouse tissues from the same point of view was not feasible because of their small size. Results were obtained, however, for four rabbit tissues; liver, lung, kidney, and leg muscle. The enzyme actions of the extracts of these tissues of the adult animal changed on standing in the same general manner. Because of lack of material it was not possible to determine the final types of the actions except for the rabbit liver, but the trend of the changes was the same for all and in a direction away from the embryo type. This fact was unmistakable. This raises the question whether tumor growths of tissues of the rabbit would not be similar to the types of actions toward which the rabbit tissue extracts were tending. No rabbit tumors, either benign or malignant, were available to test this question.

The results obtained with human uterine fibroids and human uterine muscle may be quoted as possibly bearing on this phase of the problem. The large specific increased actions on some of the esters of a number of the Type II fibroid extracts and to a smaller

extent the uterine muscle extracts may be referred to incidentally, but do not bear directly upon the point under discussion. The Type I fibroid extracts gave types of actions similar to the so called embryo type. On standing, the absolute actions of these decreased, but the type remained unchanged. The Type II fibroid extracts showed an entirely different type of enzyme actions, whose absolute values did not decrease even after very long times of standing, and whose type became constant after some weeks. The uterine muscle extracts initially showed a type of action somewhat like that of the Type II fibroid but with some definite differences. On standing, the type of action of the uterine muscle extracts changed and became much like that of the Type II fibroids after standing.

If the change on standing of the human uterine muscle type of action could be carried over to the rabbit tissues, it would be expected that some, at any rate, of the rabbit tumors would show types of actions corresponding to the type found for the rabbit tissue extracts on standing. This carrying over of the conclusions obtained with one species to another is, of course, to a certain extent hypothetical, and must ultimately be justified by the facts. For the present, therefore, the conclusions may be accepted as interesting and possibly suggestive.

One relation, however, stands out clearly. There is a fundamental difference between the embryo and the tumor types. With embryo rats and certain rat tumors, and with some human embryonic tissues and certain human tumors, the types of the enzyme actions were found to be practically identical. The further study of tissues of other animals and of a number of other kinds of tumors showed differences not only in the types of actions of the embryonic tissues and the tumors, but also in their behavior as presented in this series of four papers. The question may be raised whether the similarity of certain embryos and tumors may not be considered more or less fortuitous and due in part to the limited methods of study, and whether the differences in properties found in the other cases may not represent a truer aspect of the relations. The experimental evidence appears to the writers to point in certain definite directions, but considerable further work will be necessary to decide even the simpler questions involved.

It may be stated that a number of additional tissue and tumor

extracts was tested in connection with the changes on standing in the ester-hydrolyzing actions. Many of these showed no changes in types of relative actions, and therefore add nothing constructive to the results already presented. With some of the tissue and tumor extracts studied, too few results were available either because of the lack of sufficient material for the desired number of repetitions of the tests, or because too few of the individual tissue or tumor specimens were available to warrant their inclusion in detail. However, the results obtained with this material fall in with and corroborate in every particular the experimental data which have been presented.

#### SUMMARY.

The changes on standing in the ester-hydrolyzing actions of the extracts of the liver, lung, kidney, and leg muscle of the rabbit were studied by the method described in the preceding papers. The relative ester-hydrolyzing actions of the extracts of the tissues of the adult rabbit were shown to change in the same general way, in a direction away from the embryonic type. A number of the detailed changes in the relative and absolute actions was given.

The general significance of the changes in the enzyme actions on standing of the extracts of the different tissues and tumors which were described in this series of four papers was discussed. The difference between the embryonic and tumor actions as developed by means of the pictures of the relative ester-hydrolyzing actions was especially emphasized.





## STUDIES OF THE THYROID APPARATUS.

### XLIV. THE RÔLE OF THE THYROID AND PARATHYROID GLANDS IN THE CHEMICAL DIFFERENTIATION OF BONE DURING GROWTH. (ASH, ORGANIC MATTER, AND WATER.)\*

By FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

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A study of the chemical differentiation of the whole humerus and femur, as representatives of the skeletal system, under conditions of thyroid and parathyroid deficiency, is a part of the investigation of the rôle of these glands in the differential development of the mammal, with respect to age, sex, and interorgan relations. The results which are reported in this and the succeeding paper are analyses of the bones of the albino rats, the body and organ growth of which, and their controls, up to 150 days of age after thyroparathyroidectomy and parathyroidectomy at 23, 30, 50, 65, 75, and 100 days of age, has been the subject of earlier reports.

The details of the scope of the investigation, the procedure, methods of analysis of the data, methods of control, and the number and distribution of the animals are referred to elsewhere (1). The rats were all of the same stock, the descendants of two pairs. They were kept under the uniform conditions of diet and environment described by Greenman and Duhring (2). They were healthy and free from vermin, unmated, and the controls were litter mates of the tests.

The methods of preparation and analysis of the bones are given in an earlier report (3). Attention is called to the fact that the consistency with which the analytical results follow the age, sex, and systemic differences in bone growth in weight, is an index of

\* A part of the material given in this paper was presented before the National Academy of Sciences at the Philadelphia meeting, November, 1926. The abstract is published in *Science*, 1926, lxiv, 531.

TABLE I.  
*Mean Observed Amount of Ash, Organic Matter, and Water of Whole Fresh Humerus and Femur of Thyroidless Rats and Their Controls at 150 Days.*

Age series.	Ash.		Organic matter.		Water.	
	Control.	Test.	Control.	Test.	Control.	Test.

Male humerus.						
days	gm.	gm.	gm.	gm.	gm.	gm.
23	0.1267±0.0063	0.0955±0.0050	0.0649±0.0023	0.0549±0.0020	0.0919±0.0026	0.0848±0.0024
30	0.1302±0.0025	0.0885±0.0042	0.0638±0.0011	0.0490±0.0020	0.0890±0.0019	0.0738±0.0029
50	0.1192±0.0045	0.0859±0.0038	0.0606±0.0022	0.0484±0.0016	0.0855±0.0030	0.0739±0.0028
65	0.1239±0.0014	0.0934±0.0023	0.0624±0.0017	0.0506±0.0013	0.0874±0.0019	0.0778±0.0039
75	0.1146±0.0047	0.0901±0.0049	0.0593±0.0019	0.0511±0.0016	0.0897±0.0032	0.0778±0.0023
100	0.1031±0.0031	0.0811±0.0033	0.0516±0.0015	0.0463±0.0014	0.0849±0.0017	0.0694±0.0018

Male femur.						
23	0.2752±0.0137	0.2045±0.0111	0.1556±0.0054	0.1344±0.0052	0.2239±0.0051	0.2048±0.0067
30	0.2806±0.0063	0.1888±0.0099	0.1493±0.0025	0.1227±0.0057	0.2108±0.0048	0.1806±0.0091
50	0.2616±0.0094	0.1874±0.0100	0.1452±0.0051	0.1116±0.0061	0.2054±0.0076	0.1749±0.0059
65	0.2749±0.0112	0.1979±0.0066	0.1445±0.0044	0.1181±0.0032	0.2041±0.0051	0.1690±0.0046
75	0.2572±0.0136	0.1897±0.0115	0.1403±0.0054	0.1214±0.0039	0.2161±0.0083	0.1785±0.0056
100	0.2140±0.0069	0.1708±0.0078	0.1222±0.0029	0.1086±0.0040	0.1939±0.0039	0.1607±0.0043

## Female humerus.

23	0.1069±0.0011	0.0770±0.0032	0.0490±0.0006	0.0414±0.0013	0.0635±0.0011	0.0523±0.0020
30	0.1105±0.0022	0.0734±0.0026	0.0502±0.0009	0.0364±0.0010	0.0638±0.0011	0.0529±0.0013
50	0.0995±0.0032	0.0658±0.0028	0.0468±0.0009	0.0372±0.0013	0.0611±0.0014	0.0536±0.0021
65	0.1015±0.0017	0.0777±0.0031	0.0473±0.0007	0.0390±0.0014	0.0605±0.0010	0.0508±0.0012
75	0.0916±0.0024	0.0642±0.0033	0.0472±0.0009	0.0391±0.0012	0.0657±0.0010	0.0606±0.0023
100	0.0962±0.0034	0.0702±0.0020	0.0472±0.0014	0.0394±0.0010	0.0657±0.0017	0.0570±0.0013

## Female femur.

23	0.2308±0.0036	0.1570±0.0085	0.1140±0.0018	0.0974±0.0032	0.1581±0.0036	0.1309±0.0051
30	0.2363±0.0053	0.1500±0.0052	0.1175±0.0022	0.0855±0.0019	0.1524±0.0030	0.1300±0.0030
50	0.2137±0.0076	0.1322±0.0065	0.1086±0.0027	0.0864±0.0028	0.1465±0.0030	0.1174±0.0047
65	0.2173±0.0056	0.1621±0.0074	0.1080±0.0027	0.0904±0.0034	0.1564±0.0093	0.1192±0.0036
75	0.1952±0.0053	0.1320±0.0084	0.1099±0.0019	0.0869±0.0037	0.1554±0.0028	0.1349±0.0037
100	0.2006±0.0085	0.1519±0.0176	0.1088±0.0033	0.0939±0.0025	0.1521±0.0039	0.1343±0.0037

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their essential validity. With the exception of a few bones in the 100 day old series, all the analyses were done by one man with uniform technique. He did nothing else. Seasonal influence is eliminated by the indiscriminate collection of the material over  $5\frac{1}{2}$  years. About 16,000 separate analytical results from upwards of 1400 bones serve as the basis of these reports. It is impossible and unnecessary to record the raw data here. They are on file at The Wistar Institute. Only those essential for adequate interpretation will be given.

### *Influence of Thyroid Deficiency.*

For the reasons and with the limitations given elsewhere (1) the growth sequelæ of thyroparathyroidectomy will be interpreted in terms of thyroid deficiency alone. As these data will show, the specification is safely taken. No justification is had, however, to extend the distinction to animals other than the albino rat. On the other hand, these results should serve as a danger signal to those who are laboring to build up a theory of parathyroid function in mineral metabolism from observations made on thyroparathyroidectomized animals.

The mean observed amounts of ash, organic matter, and water of the whole fresh bones of the several groups at 150 days of age are given in Table I. The bone weights, and the observed values from bones of animals of like stock and sex of the same initial ages as the experimental series are found in other papers (4, 5). From these were computed the adjusted values of the bones of the experimental groups as of the age at time of glandular removal. This was done on the percentage basis, for it was assumed that the percentage composition of the bones of the experimental groups at this time would be the same as that of the analyzed bones from rats of the same stock and sex, raised under identical conditions. Justification for the assumption is had from the fact that the order of magnitude of the probable error of the mean percentage values is low, as can be seen in Table III. From the initial absolute amounts so calculated, and the observed terminal values, the percentage rate of increments was computed, and from these the increment capacity of the tests in terms of that of their controls. These latter figures are given in Table II.

TABLE II.  
*Percentage Rate of Growth of Fresh Humerus and Femur in Ash, Organic Matter, and Water of Testis in Terms of That of Their Controls.*

Age series.	Ash.				Organic matter.				Water.			
	Male.		Female.		Male.		Female.		Male.		Female.	
	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.
Thyroidless groups.												
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	75.7	75.8	70.9	68.0	84.5	87.8	82.2	85.3	91.4	91.9	64.4	75.9
30	65.4	66.0	62.6	61.1	71.9	79.7	63.6	66.5	65.0	77.5	46.1	70.4
50	66.1	67.1	54.7	51.7	71.4	68.7	69.4	67.9	62.4	65.9	23.2	25.8
65	60.0	57.4	64.6	63.2	61.9	63.8	64.0	69.3	47.1	24.4	-79.0*	-94.6
75	59.4	54.6	36.8	34.9	68.0	71.4	59.2	52.7	36.2	34.6	38.2	14.4
100	37.4	41.6	-1.2	6.9	60.1	64.0	25.0	49.6	-39.6	-37.0	-67.3	-38.0
Parathyroidless groups.												
23	66.1	65.7	70.7	72.9	69.9	69.6	76.6	79.6	86.8	85.4	106.8	108.2
30	71.5	74.4	84.8	87.6	71.0	76.7	85.6	90.4	84.3	87.9	121.2	109.6
50	72.5	74.8	70.4	73.6	72.2	73.7	62.6	70.5	78.6	83.7	102.9	95.9
65	56.0	56.4	81.4	86.7	50.1	52.1	79.6	83.9	27.4	30.2	275.0	384.4
75	38.8	40.5	70.4	66.2	46.2	47.4	82.6	73.7	25.4	30.2	118.3	99.7
100	67.9	73.7	27.3	32.0	75.3	72.9	48.9	44.3	16.2	21.9	78.0	84.1

\* Water lost instead of gained in control series. In test series the water loss was 79 times the percentage loss in the controls.

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They serve as the basis of the interconstituent, interage, intersex, and intersystemic comparisons.

From Table I it is clear that the weight of ash, organic matter, and water in the bones of the thyroidless rats is less than in the controls. This direction of difference is like that obtaining in bone weight (4). It indicates that all three constituents participate in the growth retardation. This unanimity is neither necessary nor predictable as will be shown.

Space restriction prevents visualization by charts of the relations brought out by the figures in Table II.

Bones grow in weight by increments of water, organic matter, and ash (5). In conditions of thyroid deficiency the processes concerned in increment by water are most affected, those concerned in ash somewhat less, and those concerned in organic matter the least. This is shown by the fact that the order of increasing degree of retardation of increment capacity is generally: organic matter—ash—water.

Before going on to an analysis of these differences, the ground will be cleared of other matters.

First as to the influence of the age of the animal. In general there is an increase in the degree of retardation with the increase in age at the time of glandular removal. Ash and organic matter pretty generally follow bone weight which follows body weight (4). This indicates that the reduction in the effectiveness of the growth processes of the body as a whole is the larger factor in the age difference reaction rather than any specific growth relation to thyroid function as such. Water presents anomalies in certain series such as actual decrements or loss of water, instead of increments. This is to be remembered. The degree of divergency of water from ash retardation tends to increase with the increase in age at time of thyroid removal. The pubertal adjustment brings about a like change in the case of ash and organic matter. This increase in disturbance of chemical differentiation with advancing age is consistent with the theory developed in an earlier paper with regard to the rôle of the thyroid in growth (1).

As noted above, the pubertal adjustment brings about a shift in response to thyroid deficiency. This is consistent with all that has come out with regard to the several organs. It is also consistent with normal bone differentiation (5). It is particu-

larly noticeable in the case of water, which again is linked with the normal bone changes.

Turning now to the influence of sex, we find that as with the bone in weight (4), so with ash, organic matter, and water, femaleness determines a generally greater degree of retardation. Although all three constituents participate, water is the largest single factor. In fact, when at puberty the reverse relation holds in the case of ash and organic matter, water is the sole factor. This is consistent with the findings on normal bone (5). This peculiar sensitivity of the water phase is again to be remembered, as is also the disturbing influence of puberty. It is worth while calling attention to the fact that this latter reaction could not have been told from the sex difference in bone weight retardation, and is only brought out by the differential analyses. The greater sensitivity of the female to thyroid deficiency is shown by all the organs. Its interpretation has been given (1). The principles established for the body hold for the chemical differentiation of the bones, as a study of the sex differences in normal rats will readily show (5).

Water is the largest single factor in the production of the systemic differences in growth retardation of bone weight (5). The average degree of systemic difference is less than the sex difference for each of the three constituents. This is correlatable with the fact that, in the normal animal, systemic is closer than sex association (6).

Returning now to the differential response of the three bone constituents, we find certain implications arise from comparing these with the degree of retardation of the bones in weight. The data for this comparison are found in another paper (4). As far as ash is concerned, the retardation is generally of the same order of magnitude as that of the bone as a whole. This suggests that ash deposition has kept up with bone growth.

Organic matter is consistently less retarded than that of the bone as a whole. Ash deposition is more retarded than organic matter increment. But ash deposition is a function of bone cell protoplasm which is organic matter. Hence increase in organic matter substance is less retarded than is its functional activity. Now Stump (7) found that growth in length of the long bones is entirely effected by cartilage increments, while Dott



TABLE III.  
*Mean Observed Percentage of Ash, Organic Matter, and Water in Whole Fresh Humerus and Femur of the Several Groups of Rats at 150 Days of Age.*

Age series.	Male.				Female.			
	Humerus.		Femur.		Humerus.		Femur.	
	Control.	Test.	Control.	Test.	Control.	Test.	Control.	Test.

Thyroparathyroidectomized. Ash.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	44.2±1.0	40.0±0.9	41.5±1.0	37.0±0.9	48.7±0.2	44.8±0.6	45.9±0.2	40.3±0.7
30	46.0±0.3	41.6±0.6	43.8±0.4	38.1±0.5	49.2±0.3	44.9±0.4	46.6±0.3	40.8±0.6
50	44.9±0.5	41.2±1.1	42.8±0.6	39.2±0.9	47.9±0.5	41.8±0.6	45.4±0.5	39.0±0.6
65	45.0±0.7	42.4±0.8	43.8±0.7	40.6±0.7	48.5±0.3	46.1±0.6	45.3±0.8	43.2±0.7
75	43.4±0.8	40.5±1.1	41.7±0.9	37.9±1.2	44.7±0.4	38.7±1.2	42.3±0.5	36.3±1.2
100	42.8±0.5	41.0±0.8	40.2±0.3	38.5±0.8	45.8±0.4	42.0±0.5	43.4±0.5	39.7±0.6

Organic matter.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	22.9±0.1	23.5±0.4	23.7±0.2	24.9±0.7	22.3±0.1	24.5±0.7	22.7±0.2	25.5±0.5
30	22.5±0.5	23.2±0.4	23.3±0.2	25.1±0.6	22.4±0.1	22.4±0.2	23.2±0.2	23.5±0.3
50	22.8±0.2	23.3±0.3	23.7±0.2	23.3±0.6	22.6±0.2	23.9±0.5	23.2±0.2	26.0±0.6
65	22.8±0.1	22.9±0.3	23.2±0.1	24.3±0.3	22.6±0.1	23.2±0.2	22.5±0.3	24.3±0.3
75	22.5±0.1	23.5±0.3	22.9±0.2	25.1±0.6	23.1±0.1	23.9±0.2	23.9±0.2	24.6±0.4
100	21.5±0.3	23.5±0.2	23.1±0.2	24.6±0.3	22.6±0.1	23.7±0.2	23.1±0.2	24.8±0.3

## Water.

23	32.9±1.1	36.5±1.7	34.8±1.1	38.1±0.6	28.9±0.2	30.7±1.0	31.4±0.3	34.2±0.8
30	31.5±0.3	35.2±0.8	32.9±0.4	36.8±0.9	28.4±0.4	32.6±0.4	30.1±0.3	35.7±0.6
50	32.3±0.4	35.6±1.2	33.5±0.5	37.4±1.3	29.5±0.4	34.3±0.8	31.4±0.4	35.0±1.1
65	32.2±0.7	34.8±0.9	33.0±0.6	35.1±0.9	28.9±0.2	30.7±0.7	32.2±1.0	32.5±0.8
75	34.1±0.8	36.1±1.1	35.4±0.9	37.0±1.2	32.2±0.5	37.4±1.3	33.8±0.5	39.1±1.3
100	35.7±0.6	35.5±0.8	36.7±0.5	36.9±0.8	31.6±0.4	34.3±0.5	33.4±0.5	35.5±0.6

## Parathyroidectomized.

## Ash.

23	45.2±0.5	39.9±0.7	42.3±0.5	37.2±0.7	48.6±0.2	41.8±0.9	45.6±0.4	38.7±1.0
30	46.1±0.2	42.4±0.4	43.7±0.3	40.0±0.5	49.6±0.2	46.3±0.5	46.6±0.2	43.8±0.6
50	45.8±0.5	42.6±0.7	43.5±0.6	40.3±0.8	47.4±0.5	43.2±0.9	45.1±0.4	41.0±0.9
65	46.4±0.3	43.3±0.5	44.9±0.3	41.3±0.6	47.8±0.6	45.3±0.6	45.8±0.4	43.3±0.7
75	44.0±0.7	38.6±0.9	42.1±1.1	36.8±1.0	44.8±0.7	41.6±0.8	43.2±0.7	39.7±0.7
100	42.8±0.5	42.2±0.6	40.2±0.3	40.4±0.6	45.8±0.4	42.2±0.6	43.4±0.5	40.5±0.7

## Organic matter.

23	22.6±0.1	21.7±0.1	23.1±0.1	22.2±0.1	22.4±0.1	21.9±0.1	22.8±0.2	21.9±0.2
30	22.7±0.1	21.7±0.2	23.4±0.1	22.8±0.2	22.3±0.1	21.6±0.1	22.7±0.2	22.2±0.2
50	22.8±0.2	22.2±0.1	23.6±0.2	22.8±0.2	23.3±0.5	21.6±0.5	23.6±0.3	22.4±0.3
65	22.7±0.1	22.1±0.1	23.0±0.1	22.7±0.2	22.6±0.1	22.1±0.1	23.0±0.2	22.3±0.2
75	22.6±0.2	22.8±0.3	22.9±0.2	23.1±0.3	22.9±0.2	23.0±0.2	23.4±0.3	23.3±0.4
100	21.5±0.3	22.3±0.2	23.1±0.2	23.1±0.2	22.6±0.1	22.7±0.2	23.1±0.2	22.8±0.2

TABLE III—Concluded.

Age series.	Male.				Female.			
	Humerus.		Femur.		Humerus.		Femur.	
	Control.	Test.	Control.	Test.	Control.	Test.	Control.	Test.
Water.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	32.2±0.6	38.2±0.7	34.6±0.6	40.6±0.7	29.0±0.2	36.4±1.0	31.5±0.4	39.4±1.1
30	31.2±0.2	35.9±0.4	32.9±0.3	37.2±0.5	28.0±0.2	32.1±0.5	30.7±0.3	34.0±0.5
50	31.4±0.4	35.2±0.6	33.0±0.5	36.9±0.7	29.3±0.2	35.2±0.7	31.3±0.3	36.6±0.8
65	30.8±0.3	34.7±0.5	32.2±0.3	36.0±0.6	29.6±0.4	32.5±0.7	31.2±0.3	34.4±0.6
75	33.4±0.6	38.7±0.9	35.0±0.7	40.1±1.0	32.3±0.6	35.4±0.7	33.4±0.7	37.0±0.8
100	35.7±0.6	35.5±0.6	36.7±0.5	36.5±0.5	31.6±0.4	35.1±0.6	33.4±0.5	36.7±0.7

and Frazer (8) report histological studies which show that the cartilage trabeculae are unduly thickened in the bones of thyroidless animals, that they fail of absorption, and that the vital activity of the cells appears to be diminished. Since growth by cartilage increments is directly a matter of cell division, the picture here presented is evidence supporting the theory that growth by increase in cell number is relatively more independent of thyroid deficiency than is growth by increase in cell mass or incorporation of new material through cell activity (1).

Water increment is generally more retarded than that of bone weight. The divergency is enhanced when the thyroid is removed during and after puberty. This is in line with the relations brought out in earlier paragraphs. In normal bone growth ash deposition is the dominant determinant of the decrease in water with age (5). It thus might be expected that in conditions of thyroid deficiency ash and water increments would run parallel courses of like intensity. That such does not occur is evidence of a dislocation of the normal relation. The comparisons just made suggest that this distortion is attributable to the special sensitivity of the water phase. A study of the percentage composition of the bones leads to interpretation.

In Table III the percentage of ash, organic matter, and water of the bones is given. The figures show that the bones of the thyroidless rats have a lower ash and a higher organic matter and water percentage than the controls of the same age.

It is results like these which have led to the conclusion that thyroid deficiency conditions a retardation of ossification. But such a conclusion is justified only on the basis of age. It fails to take account of the fact that bone growth has been retarded. It fails to recognize that the smaller bones might be merely bones at a less advanced stage of development where the ash percentage is normally less and the water percentage higher. It does not take into account the possibility that the growth retardation may have been a simple proportionate affair in which the amount of ash deposition was that consistent with the lessened total growth. It is a specious conclusion.

Now the findings with respect to the water phase, and the fact that the organic matter percentage is higher, when it ought to be lower than the control since the test bones are smaller, indicate

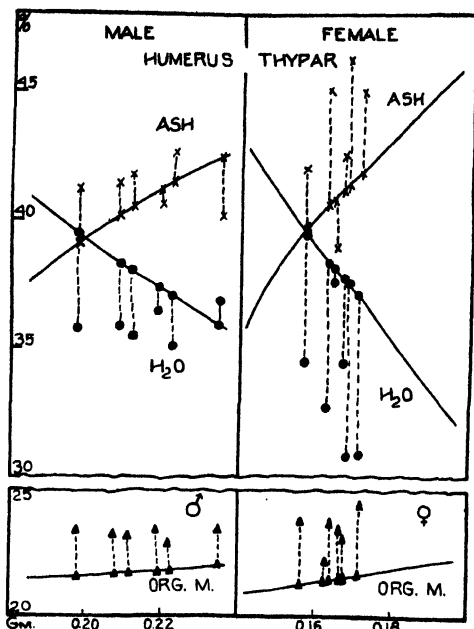


CHART 1. Comparing the observed and expected percentage composition of the humerus of the male and female thyroidless rats.

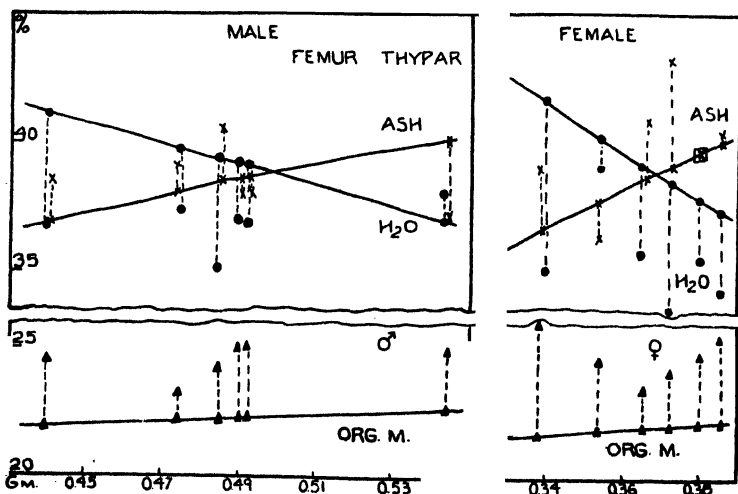


CHART 2. Comparing the observed and expected percentage composition of the femur of the male and female thyroidless rats.

that distortion of chemical differentiation has been produced. They do not show the nature of this, nor whether ossification has been less than that to be expected on the basis of actual bone size. The relative spurt in organic matter, resulting in the higher percentage, can be explained as a relative release from inhibition, in the sense and on the basis of the principle developed in the paper dealing with body growth (1).

On Charts 1 and 2, I have compared the observed with the expected percentage composition on the basis of bone weight. Percentages are plotted on the ordinates, weights on the abscissæ. The expected values are marked on the curves for the normal bone over the weight range of the group. The observed values are given by the take-offs.

Inspection shows that thyroid deficiency produces a bone of lower water, higher organic matter, and generally higher ash percentage than that to be expected in a bone of equal weight from a normal animal. If the percentage of ash is taken as the index of ossification, the conclusion must be made that thyroid deficiency does not yield a bone of a lower degree of ossification than that to be expected for the stage of development as expressed by bone weight. Consistent with this is the fact that the increment in ash is generally of the same order of magnitude as that of bone weight.

The chief factor in the distortion is the disturbance of the water phase. Now the water balance of the organism as a whole is shifted in the direction of a dehydration. This is shown by these data, by the reports published with regard to the blood (9) and central nervous system (10) of these same animals, and by the studies of Wilhelmj and Fleisher (11) and others. Significantly correlated with this appearance of a lower water content of the tissues of lowered metabolic activity is the well known fact that in normal conditions the tissues of higher water content usually have a higher metabolic activity. The conclusion seems justified that the lowered metabolic level, brought about by thyroid removal, upsets the water balance in the direction indicated, and that this starts the chain of events which produces the distortion. Hence the reaction is not due to any specific relation of water metabolism to thyroid function as such. On this basis the lesser water percentage is due to the greater retardation of the water incre-

TABLE IV.  
*Mean Observed Amount of Ash, Organic Matter, and Water of Whole Fresh Humerus and Femur of Parathyroidless Rats and Their Controls at 150 Days of Age.*

Age series.	Ash.		Organic matter.		Water.	
	Control.	Test.	Control.	Test.	Control.	Test.

Male humerus.						
days	gm.	gm.	gm.	gm.	gm.	gm.
23	0.1309±0.0042	0.0871±0.0033	0.0651±0.0018	0.0472±0.0013	0.0927±0.0024	0.0826±0.0020
30	0.1372±0.0026	0.1020±0.0032	0.0674±0.0013	0.0518±0.0013	0.0926±0.0011	0.0855±0.0016
50	0.1255±0.0041	0.0953±0.0030	0.0625±0.0019	0.0496±0.0012	0.0860±0.0028	0.0780±0.0016
65	0.1316±0.0022	0.0941±0.0034	0.0646±0.0011	0.0476±0.0015	0.0874±0.0013	0.0740±0.0017
75	0.1160±0.0046	0.0756±0.0040	0.0594±0.0019	0.0440±0.0018	0.0879±0.0028	0.0737±0.0021
100	0.1031±0.0031	0.0900±0.0033	0.0516±0.0015	0.0473±0.0015	0.0849±0.0017	0.0741±0.0017

Male femur.						
	gm.	gm.	gm.	gm.	gm.	gm.
23	0.2837±0.0096	0.1855±0.0079	0.1546±0.0043	0.1095±0.0034	0.2292±0.0044	0.1985±0.0047
30	0.2963±0.0067	0.2244±0.0078	0.1582±0.0029	0.1269±0.0031	0.2225±0.0037	0.2056±0.0042
50	0.2766±0.0092	0.2123±0.0086	0.1497±0.0042	0.1188±0.0034	0.2096±0.0067	0.1912±0.0045
65	0.2896±0.0071	0.2031±0.0093	0.1482±0.0034	0.1105±0.0037	0.2071±0.0038	0.1732±0.0043
75	0.2539±0.0125	0.1620±0.0089	0.1371±0.0049	0.0998±0.0038	0.2087±0.0069	0.1701±0.0048
100	0.2140±0.0069	0.1941±0.0085	0.1222±0.0029	0.1099±0.0038	0.1939±0.0039	0.1715±0.0043

## Female humerus.

23	0.1114±0.0011	0.0902±0.0037	0.0514±0.0011	0.0416±0.0013	0.0665±0.0016	0.0678±0.0016
30	0.1109±0.0023	0.0945±0.0020	0.0498±0.0009	0.0440±0.0006	0.0628±0.0014	0.0653±0.0012
50	0.1006±0.0023	0.0775±0.0034	0.0493±0.0011	0.0380±0.0013	0.0621±0.0009	0.0613±0.0010
65	0.1000±0.0020	0.0871±0.0030	0.0472±0.0006	0.0422±0.0010	0.0617±0.0011	0.0615±0.0010
75	0.0885±0.0034	0.0769±0.0041	0.0449±0.0011	0.0420±0.0015	0.0631±0.0010	0.0642±0.0021
100	0.0962±0.0034	0.0763±0.0026	0.0472±0.0014	0.0412±0.0015	0.0657±0.0017	0.0636±0.0027

## Female femur.

23	0.2353±0.0062	0.1723±0.0090	0.1174±0.0023	0.0959±0.0032	0.1624±0.0045	0.1689±0.0036
30	0.2373±0.0061	0.2069±0.0052	0.1154±0.0025	0.1049±0.0019	0.1562±0.0036	0.1603±0.0037
50	0.2128±0.0052	0.1666±0.0090	0.1110±0.0020	0.0993±0.0030	0.1471±0.0023	0.1433±0.0030
65	0.2090±0.0037	0.1881±0.0070	0.1052±0.0014	0.0959±0.0022	0.1423±0.0013	0.1468±0.0024
75	0.1874±0.0074	0.1577±0.0080	0.1005±0.0023	0.0907±0.0028	0.1439±0.0042	0.1442±0.0042
100	0.2006±0.0085	0.1626±0.0061	0.1058±0.0033	0.0912±0.0029	0.1521±0.0039	0.1478±0.0068



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ment capacity, and not to a replacement of water by increased ash deposition. This is supported by the fact that the usual reciprocal relationship between water and ash percentage is not maintained. It is shown in the charts where the two components do not consistently deviate from the expected in opposite directions as they should do if the normal ash-water relations held.

Attention is called to the fact that the extent of the deviations is greater in the female. This is the direction of difference generally exhibited and sustains the generalization that the female is the more sensitive to thyroid deficiency.

### *Influence of Parathyroid Deficiency.*

The mean amounts of ash, organic matter, and water are given in Table IV. They are less in the tests than in the controls, save in the case of the water in the females where they are higher in certain groups. This indicates that, with the exceptions noted, all three constituents contribute to the lesser bone weight obtaining in the parathyroidless rats. The exception shows the futility of prediction in these matters.

From Table II of the relative percentage rates of increment it is seen that retardation is general save in the water in the females.

The influence of the age of the animal at time of parathyroid removal, obtained by comparing the figures in Table II with like data for bone or body weight (4), is found to be generally the same as that exerted on the latter, save in the case of water in the males. It indicates that, with this exception, the effectiveness of the growth processes of the body as a whole is a larger factor in the determination of the age differences in response than any specific relation to parathyroid function as such.

Femaleness tends to produce a greater resistance to increment retardation of ash and organic matter. The difference is, however, not constant. Puberty in the males introduces a sex-specific distortion. The increment in water is retarded in the males, but accelerated in the females.

Systemic differences are small in degree, but are in general in the same direction as those for the bone in weight. All three constituents contribute to the greater tendency of the humerus to weight retardation. Systemic differences are less in degree

than sex differences. Hence parathyroid deficiency does not disrupt the normal associations in this respect (6).

An outstanding fact is the marked sex disharmony in differential response of the three constituents. Consistent expression has been had that puberty in the male rat conditions a sex-specific increased sensitivity to the growth-inhibiting influence of parathyroid deficiency. This disturbing influence is extended to the chemical differentiation of the bones. For whereas the relations of the increments in ash and water to those of the bone as a whole are the same in the female as in the male in the groups operated upon before puberty, in the groups operated upon after puberty the relations in the male are reversed and thus become the opposite from those of the female which remain the same throughout. I would prefer not to go into this intricacy, but its constant occurrence makes it too significant to be passed over. Perhaps a concise statement of the detailed differences will help.

In the female the retardation of ash is greater than that of bone weight. This, by the way, indicates that ash deposition has not kept up with bone growth. In the male the same holds true in the groups operated upon before puberty and the reverse in the other groups. In the female water increment is greater than that for bone weight at all ages. In the male this holds for the groups operated upon before puberty. In those operated upon during and after puberty water is more retarded than bone weight. Attention should be called to the fact that the ash relations to bone weight increase are the opposite of those for water at all times. This suggests that parathyroid deficiency has not disrupted the normal ash-water reciprocal relationship. The direction of difference between organic matter and bone weight increment is inconstant. In the female the increment in water is always greater than that in ash or organic matter. In the male this holds for the groups operated upon before puberty. In those operated upon after puberty water increment is less than that of ash or organic matter. The marked effect of sex-specific influences in the male is thus evident. It is also expressed in the percentage comparisons as will be shown presently.

Particular note should be made of the acceleration of water increment in the bones of the females. This is brought about by parathyroid deficiency and is the opposite of the tendency towards

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blood concentration observed by Collip (12) by the opposite procedure of parathyroid administration.

Turning now to the values in Table III it is seen that in general the percentage of ash and organic matter is lower, while that of water is higher in the bones of the tests. Since these bones are smaller than those of the controls and presumably at an earlier stage of development, the differences are those to be expected.

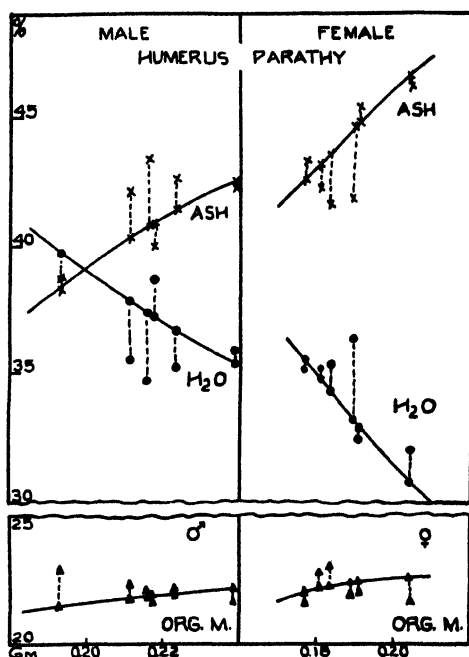


CHART 3. Comparing the observed and expected percentage composition of the humerus of the male and female parathyroidless rats.

The conclusion is that parathyroid deficiency conditions an age retardation of ossification. An exception is the bones of the 100 day old series.

On Charts 3 and 4 the observed percentages are compared with those to be expected on the basis of actual bone weight, taken as an index of the stage of development.

Inspection shows that parathyroid deficiency produces a dis-

tortion of the chemical differentiation of bone. This distortion is confined to the ash and water fractions. The organic matter percentages are to all intents and purposes those of normal bones of equal weights. The direction of deviation of ash is the opposite from that of water. Hence the normal ash-water reciprocal relationship is maintained. This, taken together with the fact that calcium metabolism is related to parathyroid function, leads to the assumption that the initiating factor in the distortion is

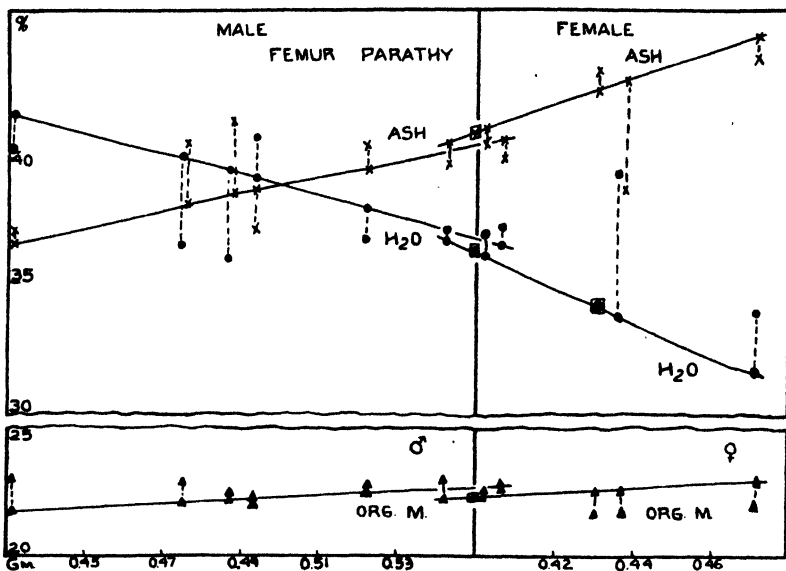


CHART 4. Comparing the observed and expected percentage composition of the femur of the male and female parathyroidless rats.

the disturbance in the calcium exchange, brought about by the glandular deficiency. While it is possible that a disturbance in water balance may be a participating factor, adequate evidence is not at hand.

The nature of the distortion differs in the two sexes. The difference is real and not methodological as a study of the body and bone weight associations, in their relations to the data in this paper, shows. In the female the distortion is towards a higher water and a lower ash percentage. In the male it is

towards a lower water and a higher ash percentage. The difference is determined by the sex-specific sensitivity of the male during the pubertal adjustment. This is a matter of degree rather than of kind for when the parathyroids are removed from the female during the pubertal adjustment ash percentage tends to be higher and water percentage lower, just as in the males of all groups operated on after the onset of the developmental change.

Of interest is the finding of Toverud (13) of a higher ash percentage in the humerus of male parathyroidectomized rats. The number of his animals and their controls were inadequate as he himself recognized. My data establish the trend as real.

Taking the percentage of ash as the index of ossification we conclude that parathyroid deficiency yields, in the female, a bone of a lower degree of ossification than that to be expected for the stage of development as expressed by bone weight, while the opposite is produced in the male.

A brief summary of the differences in reaction to the two types of glandular deficiency is indicated. Organic matter is greater than bone weight increment in the thyroidless but not in the parathyroidless rats. Water is less than bone weight increment in the thyroidless, and greater in the parathyroidless rats. Ash is of the same order of magnitude as bone weight increment in the thyroidless rats. In the case of parathyroid deficiency it is less in the females, and the males before puberty. In view of the association between parathyroid function and calcium, as a chief ash constituent, this latter difference is justification for the postulate that the *growth* changes after thyroparathyroidectomy are interpretable in terms of thyroid deficiency alone (1).

The distortion of the chemical differentiation by thyroid deficiency yields a bone of a higher organic matter percentage than that to be expected on the basis of bone weight. Parathyroid deficiency does not produce this result. Thyroid but not parathyroid deficiency, save in the males, produces a bone of lower than expected water percentage. Thyroid deficiency yields a bone of generally higher than expected ash percentage. The same holds in the case of parathyroid deficiency in the male, for the reasons given in earlier paragraphs; but in the female a lower ash percentage is the rule.

## CONCLUSIONS.

Thyroid and parathyroid deficiencies produce distinct types of distortion of the chemical differentiation of bone, with respect to ash, organic matter, and water, during growth.

Using the percentage of ash as the index of ossification, both types of glandular deficiency produce a lesser degree of ossification than that found in bones of unoperated animals of the same age. Most of this is determined by the retardation of bone growth. On the basis of the stage of development as expressed by bone weight, it is only in the case of parathyroid deficiency in the female that ossification is less in degree than normal.

Parathyroid deficiency initiated during the pubertal adjustment in the male conditions a decisive shift in chemical differentiation. This is traceable to the increased sex-specific sensitivity of the organism as a whole.

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## STUDIES OF THE THYROID APPARATUS.

### XLV. THE RÔLE OF THE THYROID AND PARATHYROID GLANDS IN THE CHEMICAL DIFFERENTIATION OF BONE DURING GROWTH. (CALCIUM, MAGNESIUM, PHOSPHORUS.)

By FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

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#### *Influence of Thyroid Deficiency.*

Extending the study of the preceding paper to include the changes induced in calcium, magnesium, and phosphorus, we secured the means given in Table I. The methods of analysis were those noted in the report on normal bone (1). The values for the percentage increment of the tests in terms of the controls are given in Chart 1. On this chart, control increase, which is always 100 per cent, is represented by the black columns, and test increment in terms of control increment in magnesium, phosphorus, and calcium by the lined columns in the order given. Comparison of these with like data for ash, the natural basis of comparison for ash constituents, yields the fact that the age, sex, and systemic relations are the same. Hence no discussion of these phases of relationship is necessary. The fact of concordance of analytical findings with ash changes puts the former on solid basis as to reliability.

Inspection of the relative degree of retardation of calcium, magnesium, and phosphorus, with respect to ash brings out significant facts. That of calcium is practically identical in degree with that of ash. This shows that calcification in conditions of thyroid deficiency keeps pace with ossification and with bone increase in weight.

Phosphorus retardation is generally less than that of ash. The same is true of magnesium, but the divergency is greater. A distortion of chemical differentiation is indicated. The order of



TABLE I.  
*Mean Observed Amount of Calcium, Magnesium, and Phosphorus of Whole Fresh Humerus and Femur of Thyroidless Rats and Their Controls at 150 Days.*

Age series.	Calcium.		Magnesium.		Phosphorus.	
	Control.	Test.	Control.	Test.	Control.	Test.
Male humerus.						
days	mg.	mg.	mg.	mg.	mg.	mg.
23	47.44±2.45	35.40±1.88	1.010±0.048	0.845±0.050	23.36±1.20	17.41±0.96
30	48.91±0.93	32.86±1.61	0.962±0.009	0.735±0.030	23.82±0.45	16.40±0.79
50	45.32±1.72	32.56±1.57	0.950±0.036	0.794±0.040	21.99±0.82	16.23±0.70
65	47.56±1.74	35.21±0.82	1.031±0.035	0.881±0.027	22.80±0.82	17.32±0.42
75	42.94±1.82	33.43±1.82	1.006±0.044	0.837±0.046	21.41±0.91	16.77±1.37
100	38.73±1.20	30.54±1.33	0.899±0.041	0.770±0.052	18.87±0.63	15.02±0.62
Male femur.						
days	mg.	mg.	mg.	mg.	mg.	mg.
23	102.92±5.30	75.81±4.14	2.197±0.112	1.867±0.120	50.29±2.57	37.40±2.06
30	105.80±2.47	70.17±5.63	2.111±0.035	1.563±0.081	51.84±1.26	37.09±1.93
50	99.24±3.57	70.74±4.08	2.102±0.102	1.719±0.106	48.65±1.92	35.29±1.88
65	104.92±4.48	74.68±2.40	2.294±0.086	1.887±0.076	50.51±2.07	37.12±1.27
75	96.57±5.24	70.45±4.29	2.250±0.120	1.751±0.110	48.31±2.36	35.72±2.23
100	80.38±2.69	63.98±3.03	1.754±0.057	1.597±0.104	39.92±1.34	31.81±1.57

Female humerus.

23	40.51±0.46	29.09±1.26	0.804±0.013	0.616±0.028	19.71±0.22	14.15±0.60
30	41.61±0.88	27.37±1.00	0.838±0.019	0.617±0.020	20.19±0.47	13.60±0.50
50	37.62±1.21	24.89±1.05	0.775±0.025	0.569±0.026	18.54±0.59	12.16±0.52
65	39.50±0.66	30.08±1.13	0.829±0.023	0.734±0.020	18.47±0.34	14.16±0.55
75	34.44±0.95	23.91±1.25	0.761±0.025	0.584±0.029	16.78±0.46	11.89±0.64
100	36.36±1.19	25.56±0.73	0.826±0.050	0.634±0.027	17.60±0.66	12.60±0.36

Female femur.

23	87.04±1.35	59.20±3.19	1.798±0.043	1.275±0.079	42.65±0.67	28.79±1.65
30	89.24±2.02	55.91±1.95	1.780±0.037	1.269±0.035	43.34±1.01	27.69±0.94
50	80.99±2.82	50.10±2.45	1.660±0.070	1.113±0.059	40.23±1.47	24.37±1.22
65	84.56±2.08	62.63±2.76	1.802±0.070	1.450±0.066	39.55±1.20	29.83±1.35
75	73.60±2.12	49.27±3.18	1.607±0.053	1.196±0.080	36.37±1.06	24.52±1.61
100	75.85±3.24	56.55±2.11	1.703±0.087	1.358±0.062	37.01±1.70	28.28±1.16

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increasing retardation is magnesium—phosphorus—calcium. As seen from Chart 1, calcium and phosphorus are closely alike in degree, while magnesium exhibits a marked deviation.

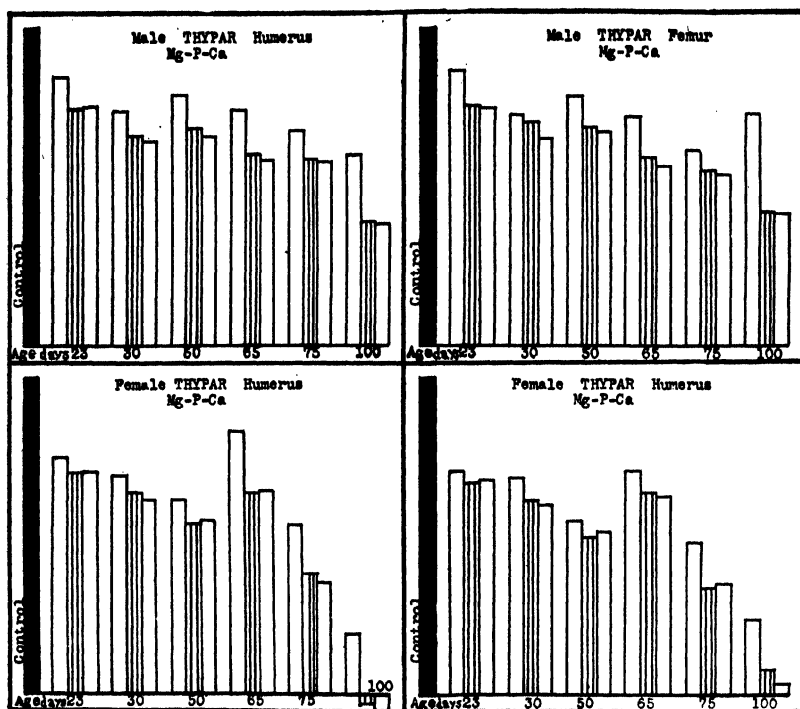


CHART 1. Relative increment in magnesium, phosphorus, and calcium in the humerus and femur of the male and female rats after thyroparathyroidectomy at each of the stated ages. The black columns represent control increment which is always 100 per cent, since the increment of each of the three constituents in the bones of the tests is computed in terms of the increment in the controls. The first column of each set of three gives the increment in magnesium, the second the increment in phosphorus, and the third the increment in calcium on this basis.

The percentage values in Table II show that thyroid deficiency yields a bone of lower calcium and phosphorus, with a trend to a higher magnesium percentage than that normal for the age. Since in normal bone (1) growth is accompanied by an increasing

percentage of all three constituents, and since these bones were retarded in increase in weight, it is clear that whereas the lower percentage of calcium and phosphorus is what might be expected, the higher magnesium is an anomaly. The direction of deviation of the three ash constituents from the expected percentage values on the basis of bone weight is generally quite the same as that for ash, as might have been predicted. The significant fact is that the degree of deviation of magnesium is much greater than that for ash, showing specific distortion response of the processes concerned with the incorporation of this element into bone during growth.

The conclusion is that thyroid deficiency does not condition a lesser degree of calcification, when the comparison is made on the basis of bone weight as the index of the stage of development.

A distortion of ash composition naturally results. As shown in Table III this is expressed by a lower than normal percentage of calcium, a higher percentage of magnesium, and a tendency to a higher percentage of phosphorus.

#### *Influence of Parathyroid Deficiency.*

The means are given in Table IV. The chief item of interest is the occasional appearance of a higher absolute amount of magnesium in the bones of the tests.

The percentage increments of the tests in terms of the controls are given in Charts 2 and 3. If these are compared with like data for ash, it is found that the age, sex, and systemic relations are the same. Hence discussion is unnecessary.

The degree of difference in retardation of each of the three elements from that of ash is significant. Calcium is consistently more retarded than is ash. This shows that in conditions of parathyroid deficiency calcification lags behind ossification. The fact supports the distinction drawn between these two processes in an earlier paper (1).

Phosphorus increment is less retarded than ash. The fact that this relation is the opposite from that of calcium suggests that either a shift has occurred in the nature of the calcium phosphate complex laid down, or that a secondary factor other than calcification is at work in the production of the phosphorus reaction. The

TABLE II.  
*Mean Observed Percentage of Calcium, Magnesium, and Phosphorus in Whole Fresh Humerus and Femur of the Several Groups of Rats at 150 Days of Age.*

Age series.	Male.				Female.			
	Humerus.		Femur.		Humerus.		Femur.	
	Control.	Test.	Control.	Test.	Control.	Test.	Control.	Test.

Calcium in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	16.51±0.41	14.80±0.32	15.50±0.40	13.71±0.33	18.43±0.07	16.94±0.27	17.32±0.09	15.19±0.30
30	17.28±0.12	15.46±0.24	16.50±0.14	14.16±0.22	18.52±0.12	16.73±0.15	17.62±0.13	15.21±0.22
50	17.07±0.22	15.61±0.48	16.23±0.24	14.77±0.40	18.10±0.22	15.83±0.24	17.22±0.19	14.79±0.23
65	17.26±0.30	16.01±0.31	16.69±0.28	15.31±0.25	18.88±0.13	17.88±0.21	17.65±0.32	16.73±0.27
75	16.25±0.34	15.02±0.42	15.64±0.38	14.07±0.47	16.74±0.21	14.43±0.49	15.97±0.22	13.55±0.47
100	16.10±0.26	15.40±0.32	15.12±0.28	14.41±0.31	17.33±0.19	15.51±0.15	16.38±0.22	14.79±0.23

Magnesium in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	0.35±0.007	0.36±0.014	0.33±0.008	0.34±0.014	0.36±0.003	0.37±0.009	0.36±0.005	0.33±0.008
30	0.34±0.005	0.35±0.006	0.33±0.005	0.32±0.007	0.37±0.004	0.38±0.006	0.35±0.004	0.35±0.005
50	0.36±0.004	0.38±0.013	0.34±0.007	0.36±0.012	0.37±0.003	0.36±0.008	0.35±0.005	0.33±0.007
65	0.38±0.006	0.40±0.011	0.37±0.004	0.39±0.012	0.40±0.005	0.45±0.023	0.37±0.011	0.39±0.012
75	0.38±0.006	0.38±0.011	0.36±0.006	0.35±0.013	0.37±0.008	0.35±0.010	0.35±0.008	0.33±0.011
100	0.37±0.011	0.39±0.019	0.33±0.008	0.36±0.015	0.39±0.015	0.38±0.011	0.37±0.011	0.35±0.010

Phosphorus in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	8.13±0.20	7.29±0.17	7.57±0.19	6.76±0.17	8.98±0.05	8.24±0.12	8.49±0.06	7.38±0.14
30	8.42±0.07	7.72±0.20	8.06±0.08	7.12±0.09	8.98±0.08	8.32±0.08	8.55±0.07	7.54±0.10
50	8.28±0.09	7.41±0.10	7.94±0.15	7.17±0.13	8.92±0.10	7.73±0.11	8.55±0.09	7.19±0.11
65	8.29±0.13	7.87±0.16	8.05±0.13	7.61±0.15	8.83±0.06	8.42±0.11	8.24±0.16	7.96±0.14
75	8.11±0.15	7.53±0.22	7.83±0.17	7.12±0.24	8.18±0.08	7.17±0.24	7.95±0.14	6.70±0.23
100	7.84±0.15	7.58±0.13	7.51±0.14	7.16±0.15	8.38±0.12	7.64±0.06	7.99±0.14	7.38±0.13

## Calcium in parathyroidectomized rats.

23	16.95±0.21	14.66±0.27	15.85±0.22	13.63±0.26	18.44±0.07	15.39±0.36	17.25±0.13	14.28±0.41
30	17.27±0.09	15.51±0.16	16.42±0.12	14.60±0.21	18.75±0.06	17.09±0.18	17.65±0.11	16.18±0.21
50	17.56±0.23	15.95±0.26	16.63±0.24	15.06±0.29	17.94±0.20	16.12±0.36	17.11±0.15	15.28±0.34
65	17.82±0.11	16.27±0.19	17.13±0.15	15.50±0.21	18.54±0.29	17.31±0.28	17.70±0.27	16.44±0.31
75	16.52±0.27	14.27±0.34	15.77±0.29	13.54±0.38	16.60±0.29	15.30±0.32	16.38±0.27	14.64±0.30
100	16.10±0.26	15.82±0.22	15.12±0.28	15.14±0.22	17.33±0.19	15.59±0.22	16.38±0.22	14.93±0.24

## Magnesium in parathyroidectomized rats.

23	0.35±0.005	0.41±0.008	0.34±0.006	0.39±0.009	0.37±0.003	0.42±0.016	0.35±0.00	0.41±0.016
30	0.34±0.003	0.43±0.005	0.32±0.005	0.41±0.007	0.37±0.006	0.47±0.007	0.34±0.005	0.44±0.007
50	0.37±0.006	0.46±0.014	0.35±0.007	0.44±0.014	0.38±0.005	0.42±0.009	0.35±0.006	0.40±0.011
65	0.37±0.004	0.45±0.006	0.36±0.004	0.43±0.008	0.38±0.007	0.45±0.011	0.37±0.007	0.43±0.013
75	0.35±0.004	0.38±0.010	0.34±0.005	0.36±0.012	0.38±0.008	0.42±0.010	0.37±0.008	0.41±0.010
100	0.37±0.011	0.44±0.009	0.33±0.008	0.40±0.008	0.39±0.015	0.44±0.010	0.37±0.011	0.41±0.010

## Phosphorus in parathyroidectomized rats.

23	8.28±0.11	7.42±0.14	7.70±0.11	6.95±0.13	8.97±0.05	7.88±0.18	8.45±0.06	7.33±0.20
30	8.48±0.05	8.04±0.08	8.04±0.05	7.60±0.11	9.04±0.03	8.70±0.10	8.58±0.05	8.29±0.11
50	8.44±0.08	8.08±0.14	8.05±0.13	7.65±0.15	8.77±0.08	8.21±0.18	8.42±0.07	7.88±0.18
65	8.59±0.06	8.16±0.11	8.30±0.07	7.85±0.12	8.70±0.12	8.40±0.12	8.33±0.08	8.05±0.14
75	8.16±0.12	7.31±0.18	7.90±0.12	6.95±0.19	8.24±0.14	7.77±0.14	8.06±0.14	7.46±0.13
100	7.84±0.15	8.01±0.11	7.51±0.14	7.68±0.11	8.38±0.12	7.94±0.11	7.99±0.14	7.66±0.14

TABLE III.  
*Mean Percentage of Calcium, Magnesium, and Phosphorus in Ash of Humerus and Femur of the Several Groups of Rats at 150 Days of Age.*

Age series.	Male.				Female.			
	Humerus.		Femur.		Humerus.		Femur.	
	Control.	Test.	Control.	Test.	Control.	Test.	Control.	Test.

Calcium in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	37.35±0.12	37.04±0.09	37.32±0.10	37.08±0.08	37.89±0.08	37.76±0.13	37.73±0.04	37.65±0.12
30	37.56±0.04	37.09±0.12	37.70±0.04	37.11±0.11	37.66±0.06	37.27±0.10	37.77±0.09	37.25±0.10
50	38.01±0.09	37.79±0.21	37.95±0.12	37.57±0.22	37.82±0.13	37.84±0.11	37.92±0.06	37.93±0.08
65	38.31±0.08	37.75±0.13	38.10±0.11	37.78±0.10	38.93±0.08	38.77±0.12	38.95±0.14	38.69±0.13
75	37.43±0.12	37.13±0.09	37.49±0.11	37.10±0.09	37.56±0.09	37.20±0.15	37.68±0.09	37.23±0.13
100	37.52±0.14	37.55±0.12	37.53±0.12	37.39±0.09	37.80±0.12	37.28±0.11	37.76±0.10	37.22±0.07

Magnesium in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	0.79±0.02	0.89±0.03	0.80±0.01	0.92±0.03	0.75±0.01	0.82±0.02	0.78±0.01	0.82±0.01
30	0.74±0.01	0.84±0.02	0.76±0.01	0.83±0.02	0.76±0.01	0.85±0.01	0.76±0.01	0.85±0.01
50	0.80±0.01	0.93±0.02	0.80±0.01	0.92±0.02	0.78±0.01	0.87±0.02	0.77±0.01	0.84±0.01
65	0.84±0.01	0.94±0.02	0.84±0.01	0.95±0.02	0.82±0.01	1.00±0.07	0.82±0.02	0.91±0.02
75	0.88±0.02	0.93±0.01	0.88±0.02	0.92±0.01	0.83±0.02	0.92±0.01	0.82±0.02	0.91±0.02
100	0.86±0.02	0.93±0.03	0.82±0.02	0.92±0.03	0.85±0.03	0.92±0.03	0.85±0.02	0.89±0.02

Phosphorus in thyroparathyroidectomized rats.								
days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	18.39±0.09	18.23±0.06	18.24±0.08	18.26±0.07	18.45±0.06	18.38±0.07	18.49±0.07	18.30±0.13
30	18.30±0.07	18.54±0.07	18.47±0.06	18.68±0.06	18.25±0.07	18.52±0.03	18.34±0.07	18.57±0.04
50	18.46±0.08	18.92±0.06	18.56±0.11	18.85±0.06	18.63±0.05	18.47±0.04	18.82±0.05	18.43±0.06
65	18.42±0.07	18.56±0.08	18.39±0.09	18.74±0.08	18.20±0.05	18.24±0.05	18.17±0.09	18.42±0.06
75	18.71±0.06	18.60±0.03	18.78±0.06	18.78±0.06	18.31±0.09	18.48±0.08	18.61±0.08	18.50±0.08
100	18.25±0.12	18.51±0.10	18.32±0.12	18.70±0.08	18.29±0.16	18.47±0.12	18.45±0.10	18.56±0.13

## Calcium in parathyroidectomized rats.

23	37.49±0.05	36.70±0.06	37.47±0.05	36.61±0.06	37.99±0.05	36.82±0.05	37.81±0.09	36.81±0.10
30	37.46±0.05	36.53±0.07	37.57±0.04	36.53±0.07	37.80±0.06	36.90±0.06	37.90±0.07	36.97±0.04
50	38.35±0.14	37.49±0.07	38.25±0.12	37.37±0.06	37.86±0.06	37.26±0.06	37.93±0.04	37.23±0.06
65	38.39±0.08	37.61±0.10	38.19±0.11	37.54±0.09	38.76±0.15	38.15±0.15	38.65±0.14	37.98±0.11
75	37.54±0.08	36.90±0.06	37.45±0.07	36.82±0.05	37.37±0.10	36.77±0.10	37.43±0.07	36.80±0.13
100	37.52±0.14	37.49±0.14	37.53±0.12	37.47±0.15	37.80±0.12	36.98±0.12	37.76±0.10	36.92±0.08

## Magnesium in parathyroidectomized rats.

23	0.78±0.01	1.02±0.01	0.80±0.01	1.03±0.01	0.75±0.01	1.00±0.02	0.76±0.01	1.04±0.02
30	0.74±0.07	1.03±0.01	0.75±0.01	1.03±0.01	0.75±0.01	1.01±0.01	0.74±0.01	1.01±0.02
50	0.81±0.01	1.07±0.02	0.81±0.01	1.08±0.02	0.80±0.01	0.97±0.02	0.78±0.01	0.98±0.02
65	0.80±0.01	1.05±0.01	0.80±0.01	1.05±0.01	0.80±0.01	0.98±0.05	0.81±0.01	1.00±0.02
75	0.81±0.01	0.97±0.01	0.82±0.01	0.97±0.01	0.84±0.02	1.01±0.02	0.85±0.02	1.02±0.02
100	0.86±0.02	1.03±0.02	0.82±0.02	1.00±0.01	0.85±0.03	1.03±0.02	0.85±0.02	1.01±0.01

## Phosphorus in parathyroidectomized rats.

23	18.32±0.14	18.57±0.10	18.20±0.07	18.66±0.06	18.47±0.06	18.86±0.07	18.51±0.07	18.92±0.06
30	18.39±0.04	18.98±0.06	18.41±0.08	18.98±0.06	18.22±0.05	18.78±0.04	18.35±0.06	18.94±0.07
50	18.44±0.07	18.98±0.06	18.52±0.10	18.98±0.05	18.53±0.05	18.98±0.04	18.68±0.06	19.20±0.03
65	18.50±0.05	18.85±0.06	18.50±0.04	18.99±0.07	18.19±0.05	18.54±0.05	18.20±0.07	18.60±0.04
75	18.56±0.07	18.88±0.07	18.75±0.08	18.91±0.06	18.37±0.08	18.70±0.05	18.65±0.05	18.78±0.05
100	18.25±0.12	18.95±0.07	18.32±0.12	19.04±0.05	18.29±0.16	18.84±0.09	18.45±0.10	18.93±0.07



TABLE IV.  
*Mean Observed Amount of Calcium, Magnesium, and Phosphorus of Whole Fresh Humerus and Femur of Parathyroidless Rats and Their Controls at 150 Days.*

Age series.	Calcium.		Magnesium.		Phosphorus.	
	Control.	Test.	Control.	Test.	Control.	Test.

Male humerus.						
days	mg.	mg.	mg.	mg.	mg.	mg.
23	49.10±1.64	31.96±1.20	1.030±0.037	0.889±0.038	24.00±0.82	16.22±0.65
30	51.37±0.95	37.33±1.20	1.019±0.019	1.041±0.034	25.23±0.48	19.36±0.62
50	48.12±1.58	35.74±1.12	1.014±0.034	1.032±0.045	23.15±0.78	18.12±0.60
65	50.54±0.89	35.34±1.48	1.069±0.020	0.972±0.044	24.37±0.45	17.77±0.77
75	43.59±1.77	27.92±1.52	0.933±0.036	0.740±0.042	21.51±0.90	14.27±0.77
100	38.73±1.20	33.62±1.19	0.899±0.041	0.921±0.033	18.87±0.63	17.05±0.60

Male femur.						
23	106.36±3.72	67.89±2.90	2.259±0.085	1.946±0.093	51.67±1.84	34.65±1.53
30	111.35±2.58	81.95±2.87	2.221±0.054	2.318±0.085	54.49±1.13	42.63±1.50
50	105.75±3.52	79.34±3.13	2.248±0.093	2.317±0.120	51.33±1.88	40.35±1.68
65	110.67±2.91	76.09±3.38	2.343±0.063	2.127±0.102	53.60±1.37	38.54±1.76
75	95.12±4.78	59.74±3.58	2.080±0.102	1.580±0.099	47.55±2.30	30.64±1.81
100	80.38±2.69	72.57±3.10	1.754±0.057	1.929±0.084	39.92±1.34	36.90±1.68

Female humerus.						
23	42.31±1.05	29.56±1.40	0.837±0.022	0.813±0.048	20.56±0.51	15.14±0.72
30	41.94±0.87	34.85±0.73	0.831±0.020	0.956±0.020	20.22±0.44	17.75±0.39
50	38.09±0.88	28.89±1.27	0.798±0.020	0.748±0.033	18.63±0.39	14.71±0.64
65	38.77±0.85	33.27±1.17	0.798±0.021	0.861±0.031	18.20±0.41	16.14±0.55
75	33.11±1.32	28.35±1.59	0.747±0.031	0.776±0.046	16.28±0.66	14.38±0.77
100	36.36±1.19	28.23±1.01	0.826±0.050	0.789±0.032	17.60±0.66	14.40±0.52

Female femur.						
23	88.97±2.28	63.57±3.28	1.799±0.051	1.822±0.115	43.55±1.08	32.66±1.73
30	89.99±2.35	76.49±1.91	1.744±0.046	2.090±0.058	43.76±1.16	39.19±1.01
50	80.81±1.94	60.02±3.02	1.662±0.047	1.642±0.087	39.73±0.95	32.03±1.55
65	80.83±1.54	71.51±2.70	1.719±0.040	1.869±0.077	38.04±0.72	35.00±1.32
75	70.30±2.83	58.19±3.09	1.592±0.071	1.609±0.087	34.98±1.41	29.61±1.50
100	75.85±3.24	60.14±2.34	1.703±0.087	1.655±0.069	37.01±1.70	30.88±1.23

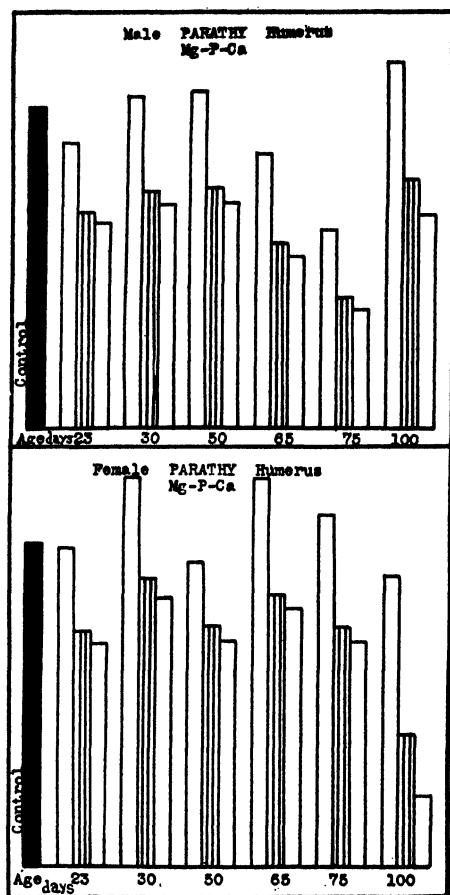


CHART 2. Relative increment in magnesium, phosphorus, and calcium in the humerus of the male and female rats after parathyroidectomy at each of the stated ages. The black columns represent control increment which is always 100 per cent, since increment in the tests is computed in terms of the increment in the controls. The first column of each set of three shows the increment in magnesium, the second the increment in phosphorus, and the third the increment in calcium on this basis.

evidence points towards the phosphorus content of the organic matter as the important factor. This includes both the cartilage and the bone marrow cells of high nuclear activity. The relatively

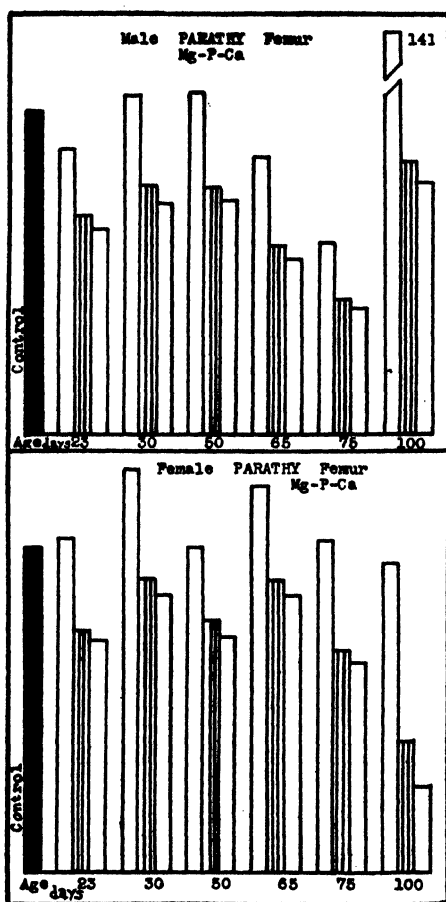


CHART 3. Relative increment in magnesium, phosphorus, and calcium in the femur of the male and female rats after parathyroidectomy at each of the stated ages. The black columns represent control increment which is always 100 per cent, since increment in the tests is computed in terms of the increment in the controls. The first column of each set of three shows the increment in magnesium, the second the increment in phosphorus, and the third the increment in calcium on this basis.

lesser phosphorus retardation can well be an expression of a relatively greater maintenance of organic matter increment, since growth by cell division is more resistant to the fundamental

metabolic upset, as shown in our earlier paper (2). Consistent with this interpretation is the fact that in conditions of parathyroid deficiency ash tends to be more retarded than bone weight, while organic matter response is generally of the same order of magnitude. The same would hold true for the lesser relative retardation of phosphorus in the bones of the thyroidless rats, where indeed organic matter increment is less retarded, and ash tends to be of the same order of magnitude as that of bone weight.

Magnesium is not only less retarded, it is even accelerated in certain groups. The order of increasing retardation is: magnesium—phosphorus—calcium. As seen in Chart 2, magnesium is the element of greatest deviation, calcium and phosphorus showing similar degrees of retardation. These findings, and the like ones reported for the thyroidless rats, are consistent with the results on normal bone, where it was seen that calcium and phosphorus tend to run parallel courses of like intensity, while magnesium diverged not only from them but from ash, during the course of bone growth (1).

The figures in Table II show that parathyroid deficiency yields a bone of lower calcium and phosphorus and higher magnesium percentage than that of normal bone from animals of the same age. Here, as in the thyroidless groups, magnesium presents the anomalous reaction.

Although calcification lags behind ossification, the percentage of this element in the bones of the tests deviates from the expected values on the basis of bone weight in the same direction as ash. Hence the discussion and the interpretation developed around the ash response holds for calcium when the bone weight or stage of development and the percentage of calcium as measure of calcification are taken as indices. On that basis it is only the bones of the females that show defective calcification.

But percentage values are the end-results of a variety of interacting factors. While these have been of such a nature as to give the picture described and to justify the conclusion drawn on the specified basis, the whole story is not told by them. For the increment capacity values show that calcification consistently lags behind ossification in conditions of parathyroid deficiency. This is all that is sufficient and necessary to show that the processes concerned in calcium incorporation into bone are specifically

retarded by the glandular removal. That this is not shown in the percentage composition is due to the fact that the growth retardation induced by the nutritional defect (2) so alters the differential development of the bones in gross that the specific response of the calcium fraction is masked. The whole thing shows, as pointed out elsewhere, that when a process is being studied, the relative progress as well as the end-result relations must be taken into account.

Reverting to the comparison of the observed with the expected percentage values, we find of importance the fact that magnesium, consistently in both sexes and regardless of the direction of deviation of ash or calcium, is higher in the tests than in the control bones of equal weight. The degree of difference is also markedly greater than that of either calcium, ash, or phosphorus.

From Table III it is seen that the ash of the tests has a higher percentage of phosphorus and magnesium and a lower percentage of calcium than that of the controls of the same age. Here too the relative distortion is greatest in the magnesium fraction.

It is clear that since the distortion of magnesium is relatively much greater than that of calcium, phosphorus, or ash, parathyroid deficiency has a greater disturbing influence on the processes concerned in magnesium metabolism, in so far as bone is concerned, than on that of calcium. This fact, which has not been generally recognized, perhaps because of the inadequacy of method and also because of the quantitative supremacy of calcium, cannot be too strongly emphasized. Any attempt to interpret mineral metabolism and also even tetany on a basis of parathyroid function, without taking cognizance of the remarkable reaction of magnesium, is bound to be incomplete.

I have no solution to offer for this complex problem. Certain consistencies appear which will be of assistance in the final untangling. The direction of shift in percentage of calcium and phosphorus previously noted (3) and tentatively confirmed by Toverud (4) is here established as general and real. It is in essence the same as that found in the blood of thyroparathyroidectomized animals by Greenwald (5). In this respect my data increase the facts from which the ultimate solution will evolve. They also add the fact of the participation of magnesium; the fact that parathyroid deficiency over long periods does not induce decalcifica-

tion of the bones, even if it does of the teeth (6), and the fact that the sex and stage of physiological development of the animal with respect to puberty must be taken into account.

Turning now to the differential response to the two types of glandular deficiencies, one can see from the percentage values in Tables II and III, that the bones of the thyroparathyroidectomized rats generally, but not always, deviate from their controls in the same direction as do those of the parathyroidectomized. This identity of response is not due to the fundamental similarity in the basis of the growth retardation (2), because if this were so the deviation would be greater instead of less in the thyroidless rats where the growth retardation is generally greater. The tables show that here the degree of divergency of the percentage values is not only less, but also inconstant. The conclusion then is that the distortion of the percentage composition of the bones and ash with respect to calcium, magnesium, and phosphorus in the thyroparathyroidectomized rats is an expression of the specific influence of parathyroid deficiency on mineral metabolism.

This might be taken as against the assumption that the growth response after thyroparathyroidectomy is interpretable in terms of thyroid deficiency alone, as developed from the accumulated data in the paper on the rôle of the thyroid apparatus in growth (2). The principle as there stated is: "the lower metabolic level due to thyroid deficiency keeps the rate of production of the toxic substances due to parathyroid deficiency down to the point where their characteristic influence is obliterated." While it is true that the characteristic influence of parathyroid deficiency is traceable in the percentage shifts in calcium, magnesium, and phosphorus of the bones, the data presented in the preceding paper and in this indicate that in so far as *growth* is concerned the primary distinction is pragmatically valid. Thus the deviation of the increment capacity of the three elements from the ash is much less in the thyroparathyroidectomized groups. The two groups differ with respect to the increment capacity in calcium, magnesium, and phosphorus in the same direction as the ash, bone weight, and body weight. This indicates that these differences are determined by the difference in degree of body growth retardation induced by the two types of glandular deficiencies. Moreover calcium increases at the same rate as ash in the thyroparathyroidectomized

groups, but falls consistently below in the parathyroidectomized. This is specific proof that the characteristic influence of parathyroid deficiency on bone growth by increments of the dominant mineral element is obliterated when the thyroid is also lacking. This finding sustains the principle and the conclusion drawn therefrom.

In this connection attention is called to Toverud's (4) correction of my interpretation of the fact that decalcification of the teeth follows parathyroidectomy but not thyroparathyroidectomy (6). This was a tentative interpretation and was modified in a later publication (2) as given above. Apparently Toverud did not have available this final analysis at the time of preparation of his monograph, for his interpretation is in essential agreement with the principle I developed from the study of the increase in body weight. I cannot, however, agree with Toverud in detail, nor will I offer a detailed explanation of my own, for it is evident from the reports of Greenwald and Gross (7-9) that the mechanism of parathyroid influence on calcium exchange is as yet unclarified.

#### CONCLUSIONS.

A distortion of chemical differentiation of bone with respect to calcium, magnesium, and phosphorus follows thyroparathyroidectomy and parathyroidectomy in albino rats. The nature of the distortion is the same in both groups, but less in degree in the former. It produces a bone of lower calcium and phosphorus and a higher magnesium percentage than that normal for the age. The ash has a higher magnesium and phosphorus and a lower calcium percentage. The shifts in the thyroparathyroidectomized groups are attributable to the parathyroid deficiency.

From the point of view of bone growth, however, the specific influence of parathyroid deficiency on calcium increment is not exhibited when the thyroid is likewise lacking.

On the basis of stage of development as expressed by bone weight the deviations from the expected percentage values are in general the same in kind as those for ash and are similarly interpretable.

Parathyroid deficiency over long periods does not result in decalcification of the bones.

A particularly important phenomenon is the relatively greater disturbance of the magnesium fraction.



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# THE SOLUBILITY OF $O_2$ , $CO_2$ , AND $N_2$ IN MINERAL OIL, AND THE TRANSFER OF CARBON DIOXIDE FROM OIL TO AIR.

BY LAWRENCE S. KUBIE.

*(From the Department of Physiology, Johns Hopkins Medical School,  
Baltimore.)*

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During the summer of 1925, in association with Dr. D. Brunswick and Dr. H. Davis of the Department of Physiology of the Harvard Medical School, an attempt was made to control the drying of mammalian nerves during studies of their electrical behavior by immersion in mineral oil. This led one of us to an investigation of the solubilities of  $O_2$ ,  $CO_2$ , and  $N_2$  in this oil, their rate of transfer to aqueous solutions, and the rate of loss of  $CO_2$  from oil to air. Although as a result of these tests, this application of the oil to nerve physiology turned out not to be feasible, the lack of any data more recent than those of Gniewosz and Walfisz (1) in 1887 and the wide-spread use of mineral oil in studies of blood chemistry warrant the publication of a part of our results.

The medicinal white oil examined was the "Amalie" brand of extra heavy medicinal white oil furnished to the Johns Hopkins Hospital pharmacy by the L. Sonneborn Sons, Inc., of New York. This oil is prepared by fractional distillation from a naphthene base, with subsequent removal of all unsaturated hydrocarbons by treatment with concentrated  $H_2SO_4$ . The product is then washed with  $NaOH$ , and decolorized with fullers' earth, bone-black, or silica gel. The resulting white oil has a specific gravity of 0.890 to 0.895 at  $15^\circ C$ . (manufacturers' determination), or of 0.868 at  $27^\circ C$ . (author's determination). Its viscosity (Saybolt) is 285 at  $100^\circ F$ . It congeals at about  $-37^\circ C$ ., is guaranteed not to cloud at a higher temperature than  $-8^\circ C$ ., and meets the United States pharmacopeial tests perfectly, in that it shows no discoloration on prolonged boiling with concentrated  $H_2SO_4$  or

HNO<sub>3</sub> and is shown to be free from S and S compounds in the PbO test.

### *I. Gas Solubilities in White Oil.*

In all of these determinations, we used the constant volume apparatus described by Van Slyke and Neill (2). To secure complete extraction of the gases from the oil in a reasonable period of time, we found it necessary to make a new calibration mark at the lowest point in the wide reservoir of the pipette, which gave our instrument a total volume of 48.37 cc. The mercury was lowered only as far as this calibration mark, so that as the pipette was shaken, a little mercury slapped backwards and forwards, beating the oil out into a thin film from which complete extraction could occur in about 10 minutes.

For transfer of oil to the Van Slyke apparatus, a pipette (with a wide bore tip) was constructed to deliver  $1.990 \pm 0.007$  cc. in exactly 60 seconds between marks. The usual pipettes could not be used because from them the delivery of the oil was too slow.

The calculation of the amount of gas dissolved in a fluid from the amount which is extracted in this instrument requires the multiplication of the actual volume of gas extracted by two correction factors. The first of these is due to the fact that some gas remains dissolved in the fluid; the second is due to the fact that in bringing the gas to the volume at which its pressure is to be measured a certain amount of gas redissolves. This latter factor is only of measurable magnitude in CO<sub>2</sub> determinations. Because we were working with an emulsion of water and oil whose solubility, for the gas, we had to consider as unknown, we could not use Van Slyke's formula for the first correction. Nor could we apply to this emulsion the reabsorption correction which he has determined on known bicarbonate solutions. Therefore, we had to determine an empirical correction for each gas, by comparing the volume of gas absorbed after a single complete extraction with that which could be obtained by extracting completely, expelling the extracted gas, reextracting the oil, again expelling, and so on, until a constant pressure reading was secured. By this method we secured a factor which combined both corrections. For CO<sub>2</sub> this factor lay between 1.07 and 1.09; and our error from this

source is thus seen to be about 2.0 per cent. For oxygen this factor was only about 1.02, and for nitrogen about 1.04, again with an error of nearly 2 parts in 100. This calculation gives the actual volume of gas per cc. of oil, reduced to standard temperature and pressure. A further correction was made for the barometric pressure under which the gas was saturated; but no corrections were introduced for the vapor pressure of oil or water, nor for the change in volume of the oil due to the gas absorbed.

Our figures indicate, therefore, the volume of gas at standard temperature and pressure which dissolves in 1.0 cc. of oil (neglecting the change of volume of the oil), under 1 atmosphere pressure of the gas (neglecting the partial pressure of water and oil vapor over the oil).

Saturation was secured by bubbling gas for several hours directly from a commercial cylinder through the oil. The oil was in a closed bottle from which the gas escaped at atmospheric pressure through an oil trap. No attempt was made to control temperature accurately; but the oil bottle and the connections were immersed in water and records were kept of the temperature both of the oil and of the bath. The constancy attained in this way is indicated in the table of solubilities given below.

*Solubility in Mineral Oil.*

	°C.
CO <sub>2</sub> at 24-25	0.841 ± 0.011
O <sub>2</sub> " 24	0.134 ± 0.004
N <sub>2</sub> " 22-22.5	0.071 ± 0.002

These solubilities may be compared, with negligible error, to the absorption coefficients (Bunsen) of these gases in water,—as tabulated in Seidell (3) from the well known investigations of Winkler, Bohr and Bock, Braun, Geffcken, and of Just.

	20°C.	25°C.
CO <sub>2</sub> .....	0.878	0.759
O <sub>2</sub> .....	0.0310-0.0317	0.0283-0.0290
N <sub>2</sub> .....	0.0154-0.0164	0.0143-0.0150

In contrast to our solubilities, Gniewosz and Walfisz (1) report the following absorption coefficients in Russian petroleum.

	10°C.	20°C.
N <sub>2</sub> .....	0.135	0.117
O <sub>2</sub> .....	0.229	0.202
CO <sub>2</sub> .....	1.31	1.17

*II. Escape of CO<sub>2</sub> from Mineral Oil to Air.*

A slow rate of diffusion of the gas through the oil is seen when oil saturated with 1 atmosphere of CO<sub>2</sub> is exposed to air. Five cups of identical cross-section and depth (*i.e.* 900 sq. mm.  $\times$  3 cm.) were set up simultaneously, and at intervals samples of oil were collected from the top and from the bottom of the cups and analyzed.

Time.	Per cent saturation.	
	Top samples.	Bottom samples.
<i>min.</i>		
5	91	
40		94
70	89	
104		93
149	70	
323	37	
358		90
403	20	

## SUMMARY.

1. The solubilities of O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub> in mineral oil are determined.
2. The extremely slow diffusion rate of CO<sub>2</sub> and presumably of other gases through mineral oil is seen to be the main protective virtue of the oil when it is used to separate a physiological fluid from air.

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## THE CHEMISTRY OF TEA LEAVES.

### II. THE ISOLATION OF GUANINE NUCLEOTIDE AND CYTOSINE NUCLEOTIDE.

BY H. O. CALVERY.

*(From the Department of Physiological Chemistry, the Johns Hopkins University, Baltimore.)*

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#### INTRODUCTION.

In an earlier paper, the preparation of adenine nucleotide from tea leaves was reported (1). It was stated there that neither guanine nucleotide, guanine, nor cytosine nucleotide had been found. That is not surprising since the methods used earlier were such that in that particular case they may have easily precluded the evidence for the presence of these substances. Modifications in the procedure have made possible the isolation of both guanine nucleotide and cytosine nucleotide.

#### DISCUSSION.

In the general procedure for the preparation of the nucleotides, the substance from which they are to be prepared is usually hydrolyzed in an alkaline medium. The hydrolysate is made slightly acid with acetic acid and the nucleotides precipitated as the lead salt by means of a neutral lead acetate solution. The lead salt is centrifuged or filtered, washed, decomposed with hydrogen sulfide, filtered, and the excess hydrogen sulfide removed by aeration. The filtrate is then evaporated to a syrup at a low temperature in a vacuum and the resulting syrup hardened with alcohol. The hardened material usually consisting solely of mixed nucleotides is then dissolved in the smallest possible amount of hot water (ordinarily 2 to 3 times its weight). The solution is then made faintly alkaline with concentrated ammonia. On cooling this becomes an opaque gel consisting of the ammonium salts of the nucleotides.

The ammonium salt of guanine nucleotide can be completely removed by addition of 1 to  $1\frac{1}{2}$  volumes of absolute alcohol. The guanine nucleotide is then prepared from the precipitate. The other nucleotides are prepared from the filtrate by fractional crystallization of their brucine salts from 35 per cent alcohol.

In the preparation of the nucleotides from tea leaves the extraction is made with dilute alkali to insure the conversion of any nucleic acid, which may be present, to the nucleotides. After straining through cloth the extract is acidified and filtered. The filtrate on treatment with neutral lead acetate gives a heavy brown precipitate which is centrifuged, washed, decomposed with hydrogen sulfide, and filtered. The filtrate is aerated, evaporated to a small volume in a vacuum at a low temperature, and hardened with absolute alcohol. This hardened material, instead of being nearly 100 per cent nucleotides as is usually the case, consists of about 10 to 20 per cent nucleotides and 80 to 90 per cent impurities. It will not dissolve in 2 to 3 times its weight of warm water but sometimes requires 15 to 20 times its weight. It was at this point that the error was made in the previous work. When an equal volume of alcohol is added a heavy black precipitate separates which consists largely of impurities. Unfortunately, it does not contain enough guanine nucleotide to enable one to detect guanine by the usual process of acid hydrolysis, precipitation with ammonia, decolorization with animal charcoal in acid solution, and final precipitation from a colorless solution with ammonia. The precipitate obtained does not contain a trace of guanine as shown by a negative murexide test. The crude adenine fraction (the filtrate above) was also tested but a negative test for guanine was obtained, no doubt due to loss in purification. Some guanine nucleotide however was undoubtedly present in the adenine fraction since the analyses of the brucine salt did not appreciably change on recrystallization from 35 per cent alcohol, a result which has recently been observed when guanine nucleotide was known to be present.

Since adenine and guanine almost invariably occur together, it seemed unlikely that it should not be so in this case. A slight modification of the above procedure changed the findings almost entirely. Instead of adding 1 or  $1\frac{1}{2}$  volumes of alcohol to a solution of the crude nucleotide in about 15 parts of water,  $\frac{1}{2}$  to  $\frac{3}{4}$

of the volume is added and the impurities, consisting chiefly of phosphates, are precipitated leaving principally nucleotides in solution. In this way about 75 per cent of the impurities are removed and then the manipulations of the nucleotides are about the same as with the mixed nucleotides obtained from yeast nucleic acid.

The crystalline guanine nucleotide obtained in this investigation was prepared by the method of Buell and Perkins of this laboratory (2). It has the characteristic crystalline structure, the same number of molecules of water of crystallization, and the same chemical composition as the crystalline guanine nucleotide, prepared from yeast nucleic acid.

The cytosine nucleotide has also the characteristic crystalline structure and same chemical composition as that obtained from yeast nucleic acid and from the pancreas.

The occurrence of the above two nucleotides in tea leaves along with the one reported earlier would seem to indicate that they are present in the form of nucleic acid. In view of the fact that nucleic acid is such an indefinite substance and that it is impossible or extremely difficult to prepare, no attempt was made to isolate it. But since the nucleotides are relatively easy to isolate and since their properties are well defined, methods were used whereby any combined nucleotides (nucleic acid) would be hydrolyzed before any attempt was made to isolate them from tea leaves. For this purpose the extraction was made with 2.5 per cent sodium hydroxide.

#### EXPERIMENTAL.

*Extraction of Tea Leaves.*—The procedure was the same as that described in the earlier paper and need not be repeated here.

*Preparation of Crude Nucleotides.*—The filtrate obtained in the procedure referred to above was slowly treated with a 25 per cent solution of neutral lead acetate until no further precipitate formed on addition of more lead acetate to a portion of the filtered liquid. About 20 cc. of lead acetate were added in excess of the above requirement and the solution was usually allowed to stand overnight. The precipitate was centrifuged from the pale yellow liquid and washed three times by grinding with cold distilled water in the centrifuge cups. The washed precipitate was suspended in



hot water and decomposed with hydrogen sulfide. The dark brown filtrate from the lead sulfide was freed from hydrogen sulfide by aeration. It was then concentrated under reduced pressure at 40–50°, to a small volume and finally in a vacuum desiccator to a thin syrup. This syrup was hardened by pouring it into 10 times its volume of absolute alcohol. The finely divided light brown precipitate, which formed, was filtered and washed twice with 100 cc. of absolute alcohol at each washing. When dry the precipitate varied in weight from 10 to 15 gm. A total of 24 pounds of tea leaves was carried through the procedure up to this point, in lots of 2 pounds each, and yielded a total of 135 gm. of crude nucleotides. The crude nucleotides were dissolved in 10 to 12 times their weight of boiling water, let stand overnight, and filtered from a small amount of insoluble material. The solution was then made faintly alkaline with ammonia and a liter of absolute alcohol was added. The dark gelatinous precipitate was centrifuged from the dark brown alcohol solution and washed once with 40 per cent alcohol. No guanine could be found in the precipitate. The combined mother liquor and washings were evaporated to dryness by means of an electric fan at room temperature. The residue was dissolved in 10 times its weight of boiling water and made distinctly acid with a very small amount of acetic acid. Neutral lead acetate was added until no further precipitate was formed when tested as above. The hot solution was cooled by allowing it to stand overnight. The lead precipitate was centrifuged from the supernatant fluid and washed three times. The washed precipitate was suspended in hot water and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated to remove the excess hydrogen sulfide and concentrated to a thin syrup by the usual process. The syrup was hardened with absolute alcohol and dried in a vacuum desiccator.

*Separation of Nucleotides into Two Fractions.*—The hardened material obtained above (20 gm.) was dissolved in 4 times its weight of warm water and made faintly alkaline with concentrated ammonia. When cool, 120 cc. of absolute alcohol were added to this solution and a heavy precipitate was obtained. It was centrifuged and washed once with 60 per cent alcohol. The precipitate constitutes the guanine fraction and the filtrate and washings constitute the adenine fraction.

*Preparation of Guanine Nucleotide.*—The guanine cake obtained above was dissolved in the smallest possible amount of hot water, cooled, treated with an equal volume of absolute alcohol, and centrifuged at high speed. This process was repeated three times. The final precipitate was dissolved in about 20 times its weight of hot water, made faintly acid with acetic acid, treated with a slight excess of lead acetate solution, cooled, and centrifuged. The precipitate was washed twice, suspended in hot water, and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated and precipitated again with lead acetate solution. The precipitated lead salt was washed three times, suspended in hot water, and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated and this time the nucleotide was precipitated as the silver salt by means of silver nitrate solution. The silver salt was centrifuged and washed two or three times with distilled water, suspended in hot water, and the silver precipitated as the sulfide by means of hydrogen sulfide. Colloidal silver sulfide may be obtained, and if so, it is almost impossible to obtain a clear filtrate. However the colloidal nature may be changed by addition of 1 or 2 drops of 10 per cent hydrochloric acid, a procedure which does not interfere with the crystallization of the guanine nucleotide. The water-clear filtrate from the silver sulfide was aerated to remove the excess hydrogen sulfide. It was concentrated in a vacuum below 40° to a small volume and then allowed to evaporate further by exposure, in an open dish, until the guanine nucleotide crystallized out. The crystals had the characteristic whetstone shape as those obtained by Buell and Perkins (2) from yeast nucleic acid.

I. Micro-Pregl for P.

5.246 mg. gave 28.06 mg. ammonium phosphomolybdate.

II. Micro-Dumas-Pregl for N.

7.474 mg. gave 1.126 cc. N at 20° and 755 mm.

	Calculated for guanine nucleotide.	Found.	
		I.	II.
P.....	7.77	7.74	
N.....	17.54		17.43

*Preparation of Guanine and Guanine Chloride.*—A sample of 182.8 mg. of the above nucleotide was hydrolyzed for 1 hour in a

test-tube immersed in a steam bath. The pale yellow solution was cooled slightly and carefully neutralized with ammonia. Further addition of ammonia was made to make a 2 per cent solution and the mixture was allowed to stand 24 hours at room temperature. The guanine was filtered on a micro asbestos filter which had been previously dried in a desiccator to constant weight. The guanine was dried similarly. Yield, 65.8 mg.

The guanine obtained above was dissolved in 15 cc. of hot 5 per cent hydrochloric acid and filtered. On cooling the characteristic macroscopic needles of guanine chloride crystallized out. They were recrystallized once from dilute hydrochloric acid and analyzed.

I. Water of crystallization.

73.8 mg. samples lost 11.9 mg. in a desiccator over sulfuric acid.

II. Micro-Dumas-Pregl for N.

3.63 mg. gave 1.009 cc. N at 28° and 755 mm.

	Calculated for $C_5H_4N_4O \cdot HCl \cdot 2H_2O$ .	Found.	
		I.	II.
Water of crystallization.....	16.10	16.12	
N.....	31.32		31.24

*Preparation of Cytosine Nucleotide.*—The adenine fraction of the alcoholic filtrate obtained above consisted chiefly of the ammonium salts of adenine nucleotide and cytosine nucleotide. This was evaporated to dryness by means of a current of air, dissolved in 3 times its weight of water, and treated with an equal volume of absolute alcohol to remove last traces of guanine nucleotide. If all the guanine was not removed this process was repeated. The precipitate which formed was filtered off and the alcoholic filtrate was treated with an equal volume of hot water, made faintly acid with acetic acid, and the nucleotides precipitated by means of lead acetate. The free nucleotides were prepared from the lead salt in the usual way and hardened with absolute alcohol. They were then dissolved in a small amount of hot water and neutralized with a hot solution of brucine in alcohol. The brucine salts were filtered off and recrystallized nine times from 35 per cent alcohol. The results of analysis after each fractionation are as follows:

Crystallization No.	Analyses. N	Calculated for:	
		Cytosine nucleotide. N	Uracil nucleotide. N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	8.27	7.92	6.79
2	7.97		
3	8.16		
4	7.84		
5	7.83		
6	7.51(?)		
7	7.82		
8	7.85		
9	7.81		

The final precipitate and the residues obtained by evaporation of the last four filtrates were combined, suspended in hot water, and an excess of ammonia was carefully added. The brucine was allowed to crystallize out overnight, filtered off, and the filtrate extracted three times with a small amount of chloroform. The solution was made faintly acid with acetic acid and lead acetate was added. The precipitated lead salt was decomposed in the usual way for obtaining the free nucleotides and instead of concentration in a vacuum the solution was allowed to evaporate spontaneously at room temperature. The characteristic crystals of cytosine nucleotide were formed.

I. Micro-Dumas-Pregl for N.

4.884 mg. gave 0.555 cc. N at 22° and 756 mm.

II. Micro-Pregl for C and H.

4.241 mg. gave 1.74 mg. water.

III. 4.241 " " 5.16 " CO<sub>2</sub>.

	Calculated for cytosine nucleotide.	I.	Found. II.	III.
N.....	13.00	13.08		
H.....	4.33		4.58	
C.....	33.43			33.20

The author wishes to express his sincere appreciation to Dr. O. Wintersteiner, Rockefeller Foundation Fellow, from Professor Pregl's laboratory at Graz, who has done several of the micro analyses reported in this paper while testing out his apparatus here. He also supervised the others.

## SUMMARY.

Guanine nucleotide and cytosinè nucleotide were prepared from dried tea leaves. Both nucleotides possessed the characteristic crystalline structure and properties of those obtained from yeast nucleic acid. It seems very probable that the nucleic acid, which is characterized by the presence of a pentose, is a naturally occurring product in tea leaves.

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## QUANTITATIVE STUDIES OF VITAMINS A, B, AND C IN GREEN PLANT TISSUES OTHER THAN LEAVES.\*

BY E. J. QUINN, M. P. BURTIS, AND E. W. MILNER.

(From the Department of Chemistry, Columbia University, New York.)

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In previous studies of the distribution of vitamins in nature, considerable attention has been given to the seeds and to the green leaves of plants and to some of the roots, tubers, and fruits, while relatively little has been known of the occurrence and quantitative concentration of the vitamins in other plant tissues. The purpose of the present paper is, therefore, to record the results of quantitative studies of the relative concentrations of vitamins A, B, and C in two green plant tissues other than leaves; namely, the green seed pod of the string bean, and the fruit flesh of the green pepper. In both cases the tissue was studied at the stage of development at which it is used as human food, the samples being obtained by purchase in the retail markets of New York City.

For the feeding tests the beans were cut in small cross sections and included not only the flesh of the pod but a section of the immature seed itself. The seeds and cores of the peppers were discarded, only the flesh of the pods being utilized as in their preparation for food.

Below are given the results obtained in the tests for the vitamins A, B, and C, together with brief references to the procedure followed in each case. For convenience the data have been summarized in Tables I, II, and III.

### *Vitamin A.*

The method of determining vitamin A and the unit expressing the results were those used by Sherman and Munsell.<sup>1</sup> After

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<sup>1</sup> Sherman, H. C., and Munsell, H. E., *J. Am. Chem. Soc.*, 1925, xlvii, 1639.

young rats were depleted of their bodily surplus of vitamin A so that growth had ceased and other signs of the lack of this vitamin were just beginning to become apparent, graded portions of the food to be tested were fed in addition to the basal diet which was deficient in Vitamin A but otherwise adequate. A unit of

TABLE I.  
*Quantitative Measurements of Vitamin A Values.*

Material tested.	Amount fed daily.	No. of animals.	Average gain in 8 wks. experimental period.	Remarks.
	gm.		gm.	
Green string	0	3	-20	Average survival 37 days.
beans. Series	0.05	2	-3	" " 49 "
I (1923).	0.09	3	-2	" " 53 "
	0.22	3	15	Killed at end of experimental period.
Green string	0	3	-24	Average survival 26 days.
beans. Series	0.05	1	-9	Survived 37 days.
II (1926).	0.09	3	28	Killed at end of experimental period.
	0.13	3	44	" "
	0.17	6	50	" "
Green peppers	0	3	-30	Average survival 28 days.
(1926).	0.05	2	0	" " 41 "
	0.09	2	28	Killed at end of experimental period.
	0.17	5	39	" "
	0.35	4	40	" "

vitamin A has been taken by the above workers to be that amount which when fed daily just suffices to support a limited gain of 3 gm. per week in a standard test animal such as they describe.

Weight curves in these experiments are believed to be determined practically entirely by the intake of vitamin A, and therefore to serve as a quantitative measure of the relative amounts

of this vitamin in the foods tested. All the rats came from stock which for generations has had a large amount of whole milk powder, or its equivalent in butter and skim milk powder, in the diet and, as other experiments in this laboratory have shown, such animals still have supplies of vitamin D stored in their bodies, since the growth of the experimental animal is intentionally retarded by such a low vitamin A intake as to make little demand on the antirachitic factor.

*Green String Beans.*—Two series of tests were made of the vitamin A content of green string beans. The first series was conducted during the summer and fall of 1923;<sup>2</sup> the second during the summer of 1926.

In Table I are given the average gains in weight of the animals fed graded portions of green string beans during the two series of tests. In 1926, 90 mg. daily produced somewhat better growth in rats than did the 220 mg. daily in the earlier experiments. On the unit basis the beans of the first series contained less than 5 units of vitamin A per gm., while beans of the second series contained over 10 units per gm. or over twice as much as those of the 1923 experiments. Whether or not the differences found can be attributed to conditions under which the beans were grown, such as soil, irrigation, or weather, it is significant that there may be considerable variation in the vitamin content of a natural product of this kind and any attempt at comparisons of foods for their vitamin A content should take cognizance of the possibility of such variations, and also of the fact that methods have now been developed which permit of their quantitative study.

*Green Peppers.*—Vitamin A in green peppers, selected as the green "fruit" to be tested, was found to be present to the extent of about 10 units per gm., since 90 mg. daily of the flesh of this fruit gave unit growth when fed to rats for the experimental period of 8 weeks.

In Table I, it will be seen that green peppers contained about twice as much vitamin A as was found in the green string beans of the first series but not quite as high an amount as found in the beans of the second. The experiments indicate that there is no marked difference in vitamin A content in the two foods examined;

<sup>2</sup> All experiments of the 1923 series were conducted in this laboratory by Dr. H. E. Munsell.



in fact there was less difference found between the pod vegetable and the pod-like fruit than was found in different samples of the same vegetable.

### *Vitamin B.*

The method of procedure in determining vitamin B was that used by Sherman and Spohn.<sup>3</sup> Rats 28 days of age were placed

TABLE II.  
*Quantitative Measurements of Vitamin B Values.*

Material tested.	Amount fed daily.	No. of animals.	Average gain in 8 wks. experimental period.	Remarks.
	<i>gm.</i>		<i>gm.</i>	
Green string beans. Series I (1923).	0	3	-12	Average survival 30 days.
	0.9	1	-17	Survived 49 days.
	1.8	3	-9	Killed at end of experimental period.
	3.5	3	0	" "
	7.0	3	41	" "
Green string beans. Series II (1926).	0	1	-4	Survived 35 days.
	1.8	2	-11	Average survival 42 days.
	2.5	2	-5	" " 49 "
Green peppers (1926).	0	4	-15	Average survival 31 days.
	1.8	2	-15	" " 42 "
	3.5	5	-4	" " 53 "
	4.5	3	1	Killed at end of experimental period.
	5.0	4	29	" "

on a diet devoid of vitamin B but adequate in all other respects, and fed graded portions of the material to be tested. It has been found that consistent results are obtained on that level of vitamin B intake producing net maintenance of weight during an experimental period of 8 weeks. The amount of food giving this result

<sup>3</sup> Sherman, H. C., and Spohn, A., *J. Am. Chem. Soc.*, 1923, xlv, 2719.

is said to contain 1 unit of vitamin B and it is this unit (suggested by Sherman)<sup>4</sup> which is used in the expression of results which follow.

*Green String Beans.*—Tests for vitamin B made at the same time and with the same supplies as the experiments of Series I on vitamin A showed string beans to contain about 0.3 units of vitamin B per gm. since daily feedings of 3.5 gm. portions of the vegetable resulted in unit growth in rats during the 8 weeks experimental

TABLE III.  
*Quantitative Measurements of Vitamin C Values.*

Material tested.	Amount fed daily.	No. of animals.	Average gain in 90 days experimental period.	Average scurvy score.	Remarks.
	gm.		gm.		
Green string beans (1926).	0	6	—125	15	Average survival 31 days.
	0.9	2	—137	12	“ “ 35 “
	1.7	2	—143	17	“ “ 60 “
	3.4	3	53	5	Killed at end of experimental period.
	4.3	1	138	0	“ “
Green peppers (1926).	0.4	2	—54	12	Average survival 60 days.
	0.9	2	265	0	Killed at end of experimental period.
	1.7	2	233	0	“ “
	2.6	2	355	0	“ “
	5.1	2	384	0	“ “

period. It was found that 2.5 gm. of beans daily was not sufficient to result in maintenance of the animals in the case of the vitamin B tests of 1926. The results of the two series of experiments, as shown in more detail in Table II, indicate that the marked higher vitamin A content of the beans tested during the summer of 1926

<sup>4</sup> Sherman, H. C., Chemistry of food and nutrition, New York, 3rd edition, 1926.

compared with those of 1923 was not paralleled by a correspondingly higher vitamin B content.

*Green Peppers.*—Judging from Table II, the green peppers contained about 0.2 unit of vitamin B per gm. but approximately the same as was found in green string beans.

#### *Vitamin C.*

The vitamin C content of the two foods was determined according to the method used by Sherman, La Mer, and Campbell,<sup>5</sup> and the unit of vitamin C, as here used, is that defined by Sherman<sup>4</sup> as "that amount which when fed as a daily allowance just suffices to afford complete protection from scurvy to a standard guinea pig," in these cases for a period of 90 days.

*Green String Beans.*—In a preliminary test of green string beans for their vitamin C content (made by Miss H. L. Campbell in this laboratory in 1921), 3.2 gm. daily protected a guinea pig from scurvy during an experimental period of 76 days. Another guinea pig receiving 2.8 gm. daily showed traces of scurvy at the end of the 76 day period.

In 1926, experiments were conducted in which the daily portions indicated in Table III were fed. 4.3 gm. of the beans daily gave complete protection from scurvy and allowed considerable growth during the experimental period. The green string beans fed contained therefore about 0.25 unit of vitamin C per gm.

*Green Peppers.*—The most striking difference in the comparisons made between the beans and peppers was in their vitamin C content. Green peppers were found to contain at least 4 times as much vitamin C as green string beans. 0.9 of a gm. of the pepper daily was found to give complete protection from scurvy and produced good growth, showing the presence of over 1 unit of vitamin C per gm. of the peppers.

#### SUMMARY AND DISCUSSION.

A quantitative study was made of the vitamin content of green string beans and green peppers.

<sup>5</sup> Sherman, H. C., La Mer, V. K., and Campbell, H. L., *J. Am. Chem. Soc.*, 1922, xlv, 165.

Both the string beans and the green peppers contain vitamin A in about the same concentration as has been found for lettuce, more than for cabbage, but considerably less than for spinach.

The vitamin B concentration of about 0.3 unit per gm. indicated that both string beans and green peppers are as good sources of this vitamin as lettuce or cabbage but not as good as spinach.

Green peppers were found to contain at least 4 times as much vitamin C as string beans or about 1 unit per gm. This concentration is comparable with the highest which has been reported for any plant material, such as the raw leaf tissue of cabbage or spinach or the juice of orange or lemon.



# THE CONDITION OF THE INORGANIC PHOSPHORUS OF THE BLOOD WITH SPECIAL REFERENCE TO THE CALCIUM CONCENTRATION.

By ARTHUR GROLLMAN.

*(From the Laboratory of Physiology, the Johns Hopkins University, School of Medicine, Baltimore.)*

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## I.

### INTRODUCTION.

The effective concentration of any constituent of the blood stream is determined not only by its total concentration as given by chemical analysis, but rather by its concentration in an active form. Hence, the condition of the constituents of the blood is of paramount importance and has been the subject of many investigations in recent years. The condition of the inorganic phosphorus of the blood has been studied by several investigators. Rona and Takahashi (1) by compensation dialysis of horse serum found the inorganic phosphorus to be completely diffusible. Cushny (2), using ox blood, also found complete filtrability, and Neuhausen and Pincus (3) by ultrafiltration of pig serum obtained a similar result. Tschimber and Tschimber (4) by ultrafiltration of serum found only 30 per cent of the inorganic phosphorus to be filtrable. Mayrs (5) reported the phosphates of the blood serum of the cockerel to be only partially filtrable. The use of high pressures and the failure to obtain complete filtrability for protein-free solutions throws doubt on his results. Recent studies by the author (6) have shown the necessity of taking certain precautions in ultrafiltration studies, which have been overlooked by previous workers. Moreover, investigation of the excretion of phosphate by the kidney has led to the view that the inorganic phosphate of normal serum may be present in a non-ionized, colloidal, and hence, non-diffusible form. It was, therefore, thought worth while to rein-

investigate this problem of the state of the inorganic phosphorus of the blood. The present paper gives the results of this study.

## II.

## EXPERIMENTAL.

The technique followed in these experiments was the same as that described in previous publications (7, 8). Preliminary tests

TABLE I.

*Diffusibility of the Inorganic Phosphorus of Various Animal Species.*

Animal.	Serum from:	Concentration of inorganic phosphorus in serum.	Per cent of inorganic phosphorus filtrable.
		<i>mg. per 100 cc.</i>	
Pig.....	Clotted blood.	8.6	100
" .....	" "	8.4	100
" .....	Defibrinated blood.	7.3	100
" .....	" "	9.1	100
" .....	Clotted blood.	9.7	100
" .....	" "	9.5	90
Dog.....	Heparinized blood.	5.4	100
" .....	Hirudinized "	5.4	100
" .....	Oxalated blood.	5.4	100
" .....	Clotted "	5.4	100
" .....	Heparinized blood.	5.0	100
" .....	Defibrinated "	3.4	100
" .....	" "	6.4	100
Chicken.....	Clotted blood.	2.0	85
Frog ( <i>Rana</i> <i>catesbiana</i> ).....	Non-coagulated blood.	5.5	85
Terrapin.....	" "	1.5	60
" .....	Clotted blood.	1.5	60
" .....	" "	1.8	90
" .....	" "	3.8	50
" .....	" "	6.5	60

were made to determine that none of the errors which were pointed out (6) as vitiating many studies by ultrafiltration through colloidion applied to the present study. Blood serum or plasma was used in all cases. This was obtained from clotted or defibrinated blood, or from blood treated as described in the protocols. In the case of the blood of the frog and terrapin, blood plasma, to

which no anticoagulant had been added, was used in several experiments. A paraffin-lined cannula was introduced into the carotid artery and the blood was received into paraffined centrifuge tubes surrounded by ice. The tube and the filter were likewise kept cold to avoid coagulation. In this way the plasma could be studied without the addition of an anticoagulant. Filtrations were carried out at a pressure of 200 mm. of mercury. The inorganic phosphorus was determined by the Brigg's modification (9) of the Bell-Doisy method. Calcium analyses were made according to Clark and Collip's modification of the Kramer-Tisdall method (10).

In Table I are given the results of twenty determinations on the serum of the pig, dog, chicken, frog (*Rana catesbiana*), and terrapin (*Pseudemys rugosa*). The fraction of the total inorganic phosphorus which is filtrable through collodion membranes is given in the last column. These values have been corrected for the protein content of the original serum. It will be seen that although the inorganic phosphorus of the mammals (pig and dog) is completely filtrable, that of the fowl, frog, and terrapin is only partially so. This species variation, as we shall see later, is explicable by a difference in the ionic calcium concentration of the blood of the different species studied.

### III.

#### *Effect of Calcium Concentration on Filtrability of Inorganic Phosphorus.*

By adding calcium chloride to serum in amounts which caused no precipitation or change in the concentration of the inorganic phosphorus, the latter was rendered non-filtrable. This is shown in the results of Table II. The same results were obtained with the sera of dog, chicken, frog, or terrapin. Addition of calcium chloride caused more of the inorganic phosphorus to become non-filtrable, while addition of sufficiently large quantities caused the complete disappearance of any inorganic phosphorus from the ultrafiltrate. The condition of the inorganic phosphorus of blood serum is thus variable and dependent on the calcium concentration. This view will explain the variation in the filtrable phosphorus with various species found in Table I. It has been demonstrated by



numerous observers that the calcium is bound, to a considerable extent, to the proteins (11). The amount of this binding is proportional to the protein concentration. Hence, relatively less calcium is bound and the amount free is greater in the lower animals and amphibia, the protein concentration of whose sera is relatively low. Moreover, the calcium concentration in the terrapin, for example, is higher and the phosphorus concentration lower

TABLE II.

*Effect of Calcium Concentration on Filtrability of Inorganic Phosphorus of Blood Serum of the Pig.*

Calcium.	Inorganic P.	Per cent of phosphorus filtrable.
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
9.4	9.7	100
10.0	10.6	95
10.0	9.5	91
13.5	9.8	80
16.0	9.7	34
19.1	8.7	22
20.6	9.3	10
32.2	9.5	5

TABLE III.

*Filtrability of the Inorganic Phosphorus and Calcium of the Blood Serum of the Terrapin.*

Serum concentration.		Ultrafiltrate concentration.	
Inorganic phosphorus.	Calcium.	Inorganic phosphorus.	Calcium.
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
3.8	13.9	1.9	8.6
6.5	12.0	3.9	6.3

than that of the dog or pig. If then there be an equilibrium between the calcium and phosphorus which determines the filtrability of the phosphorus, we would expect the latter to be less filtrable in those cases in which low protein, high calcium, and low phosphorus concentrations obtain. Such is the case in the fowl, frog, and terrapin studied in this work. Table III shows the filtrability of the Ca and phosphorus of terrapin serum. The con-

centration of filtrable Ca is such as would render the phosphorus of other sera (as in Table II) partially non-filtrable.

It is worthy of note that although the inorganic phosphorus of normal mammalian blood is completely filtrable, the concentration of calcium and phosphorus in such blood is such that, if but slightly increased, the inorganic phosphorus will no longer be completely filtrable. This is evident from the results of Table II. In conditions of hypercalcemia one must, therefore, consider the possibility of the presence of some non-diffusible inorganic phosphorus whose existence may have a bearing on certain pathological conditions.

#### IV.

#### *Effect of Parathyroid Extract on Filtrability of Inorganic Phosphorus of Dog Serum.*

In order to determine the effect of an increased calcium concentration produced physiologically, on the condition of the inorganic phosphorus, parathyroid extract was utilized to produce a hypercalcemia in dogs. The following experiment describes the results.

A well nourished male mongrel dog, weighing 15.1 kilos, was given intravenous injections of parathormone, Lilly<sup>1</sup>, a parathyroid extract prepared according to the method of Collip (12). A total of 170 units was injected in five doses over a period of 18 hours. The dog showed symptoms of dyspnea, weakness, and the "dullness, drowsiness, verging on coma, general atonia and a failing circulation," observed by Collip. 24 hours after the first injection, blood was drawn from the left heart and femoral artery. The animal appeared normal on the following day.

The inorganic phosphorus rose from 5.1 mg. per 100 cc. of blood serum (its value before the injection) to 8.7 mg. The calcium content rose from 9.8 to 17.9 mg. per 100 cc. The inorganic phosphorus which was completely filtrable before the experiment was now only 63 per cent filtrable. The calcium likewise was found to be only 40 per cent filtrable, which is lower than usually found (13). This decreased filtrability is due to the combination of a part of the calcium with the non-filtrable phosphate. Increased

<sup>1</sup> This material was obtained through the courtesy of Dr. H. W. Rhoehnanul, of Eli Lilly and Company, Indianapolis.

calcium concentration, just as in the case of the *in vitro* experiments described above, thus produced a decreased filtrability of the inorganic phosphorus of the blood. It is worthy of note that although the total inorganic phosphorus is increased, the filtrable phosphorus remains constant. The rise of the total phosphorus content which has also been observed by Collip, Clark, and Scott (14) may be attributed to an equilibrium which maintains a constant concentration of the diffusible constituent. It is only this fraction of the total which forms the available and active concentration, and hence, its constancy, rather than that of the total phosphorus, is of importance to the organism.

## V.

## DISCUSSION.

The above results must indicate not only that inorganic phosphorus may be present in the blood as a simple diffusible phosphate anion, but that it may also be present in the form of some non-diffusible complex. The dependence of the diffusible phosphorus on the calcium concentration would indicate that this complex is either a colloidal combination of calcium and phosphate stabilized by the blood proteins as in the preparations of Neuberg (15) or in actual combination with them, as hypothesized by Porcheur (16) for his  $\text{Ca}_3(\text{PO}_4)_2$ -calcium caseinate complex. A similar existence of colloidal  $\text{Ca}_3(\text{PO}_4)_2$  in milk has been previously noted (17). The fact that the non-diffusible phosphorus remains in the deproteinized plasma would indicate that, if bound to the plasma proteins, this combination must be a rather loose one, which is split off in the deproteinizing process.

In considering the inorganic phosphorus concentration of solutions it is, therefore, necessary to take into account this equilibrium between the free and non-diffusible fractions. Likewise, the existence of calcium in the form of a complex with phosphate is also to be considered in addition to the already recognized forms in which calcium exists in protein solutions. These forms of calcium and phosphorus have heretofore been neglected in many considerations in which account must be taken of them. Thus, studies on the solubility of  $\text{Ca}_3(\text{PO}_4)_2$  based on a study of the calcium and phosphate concentrations would involve a consideration of the

equilibrium between free and diffusible phosphate. The great solubility of  $\text{Ca}_3(\text{PO}_4)_2$  in blood as compared to its solubility in water may be explained partially at least by the formation of this colloidal complex combined with protein.

The bearing of the condition of the inorganic phosphorus of blood serum on the problem of renal function has been recently discussed by Marshall (18). The results of Table III showing the effect of the addition of calcium chloride on the diffusibility of the inorganic phosphorus explain the results observed by Starling and Verney (19). These investigators found that the addition of  $\text{CaCl}_2$  to the perfusion liquid in a heart-lung-kidney preparation prevented the appearance of inorganic phosphorus in the urine. The non-diffusibility of the phosphate after the addition of  $\text{CaCl}_2$  and its consequent inability to pass the glomerular membrane readily account for this result. The observation of White and Schmitt (20) on the absence of phosphate in the glomerular fluid of the mud-puppy (*Necturus maculosus*) may also possibly be explicable by a high content of non-diffusible phosphorus which is expected if we consider the position of this animal among the species noted in Table I.

## VI.

### SUMMARY.

A study has been made of the state of the inorganic phosphorus of the blood sera of various animal species. It has been shown that although the inorganic phosphate of mammalian blood is in general completely filtrable, being present as simple ionic phosphate, that of the lower animals is only partially so. This species variation is shown to be dependent on the ionic calcium concentration of the blood. Hypercalcemia as produced, *e.g.*, by the injection of parathyroid extract, causes a partial conversion of the filtrable phosphate to a non-diffusible form. The bearing of these findings on studies of the solubility of  $\text{Ca}_3(\text{PO}_4)_2$  in blood plasma and on the elimination of inorganic phosphorus by the kidney is discussed.

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## THE CHEMICAL STUDY OF BACTERIA.

### XIV. A PRELIMINARY STUDY OF *BACILLUS LACTIS AEROGENES* GROWN ON SYNTHETIC MEDIA.\*

By DONALD M. HETLER.†

(From the Department of Chemistry, Yale University, New Haven.)

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#### INTRODUCTION.

The results which have been obtained in our research in this laboratory on the tubercle bacillus, have stimulated an interest in the chemical composition of types of bacteria which are classified as non-pathogenic. It seemed of especial interest to determine whether characteristic chemical differences would be revealed if strictly pathogenic and non-pathogenic organisms were submitted to the same chemical treatment. On account of its wide distribution in nature, its practical non-pathogenicity for man, and the ease with which it can be grown in quantity, *Bacillus lactis aerogenes* was chosen as the organism to be investigated. So far as the writer is aware, no work has been recorded dealing with the question of proximate analysis of its cell constituents.

#### *Growth of the Organism.*

Very few of the bacterial organisms investigated in the past have been grown on synthetic media. Most of the media heretofore utilized for growth contained protein, peptone combinations, or simpler degradation products of proteins. For our research it was desired to be absolutely certain that any organic combination, which might be isolated from the bacterial cell during the inves-

\* Constructed from a dissertation presented by Donald M. Hetler, in June, 1926, to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy (T. B. Johnson).

† Holder of a National Tuberculosis Association Junior Fellowship in 1924-25.

tigation, could be formed only by synthesis in the cell and would not be a product absorbed from the media, or mechanically held back during the filtration of the organisms from the culture medium. The only source for nitrogen was ammonium phosphate. The media used consisted of the following composition:

	gm.
Distilled water.....	1000
NaCl.....	5.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2
CaCl <sub>2</sub> .....	0.01
HK <sub>2</sub> PO <sub>4</sub> .....	0.40
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> .....	0.60
Glycerol.....	30.0

The acidity of the culture media was adjusted colorimetrically before use, so that the pH value was between 6.8 and 7.0.

Attempts to grow the organism in bulk on solid media and in Kohle flasks were impractical. After several trials with flasks of different sizes recourse was finally taken to 5 and 10 liter flasks, and the solution was aerated during growth by bubbling a current of sterile air through the fluid. This speeded up the growth to such an extent that the pH value dropped consistently from 7 to 3.8 to 3.6 in about 6 days. Maximum growth was reached in this time and the organisms were then separated from the media by the use of a Sharples supercentrifuge. By equipping these large culture flasks with dropping funnels and adding as needed known amounts of 10 per cent sodium hydroxide solution daily to maintain a pH value between 6 and 7 it was found possible to speed up the growth of the organism and obtain a much larger yield per flask in the same length of time. After separating the bacteria from the culture medium they were suspended in cold distilled water and thoroughly washed and finally separated again by means of the high power supercentrifuge. This procedure was repeated until the wash water gave no appreciable test for chlorides. The bacteria were then either autoclaved and dried in a vacuum, or dried immediately at a low temperature. The final drying operation in all cases was conducted in a vacuum over concentrated sulfuric acid at temperatures below 35°.

By application of this technique we succeeded in growing 125 gm. of the organism which were dried without autoclaving, and

210 gm. which were autoclaved before drying, or a total of 335 gm. of desiccated cells. 85 gm. of the autoclaved product were used for the production of nucleic acid according to the procedure employed by Johnson and Brown (1). A description of this new bacterial nucleic acid will be given in a future publication.

Although time did not permit us to investigate them, advantage was taken of our opportunity to extract from the culture media the organic acids produced by the bacteria during its growth. This was accomplished as follows: The medium was made weakly alkaline with sodium hydroxide, evaporated to a syrup at 100°, and the residue dissolved in a small amount of distilled water. This solution was then saturated with sodium chloride, acidified with sulfuric acid, and the mixture finally extracted with ether in an especially devised extractor, which was so constructed that we were able to operate with large quantities of material in one unit. This mixture of organic acids obtained by ether extraction has been set aside for future research.

#### EXPERIMENTAL.

The dried organisms were pulverized in a mortar and passed through a 170 mesh sieve. The autoclaved bacteria were heated at 110° to constant weight. The unautoclaved organisms were not heated above 35°. All nitrogen determinations were made according to the Kjeldahl method, and phosphorus determinations according to the official method described in "Methods of Analysis of the Association of Official Agricultural Chemists" (2). The analytical values obtained are recorded in Table I.

In order to remove the fat and waxy constituents the autoclaved cells were extracted successively with alcohol, ether, and toluene in a Soxhlet extractor. The unautoclaved bacteria, on the other hand, were extracted with ether only, and at no higher temperature than 25°. Six successive cold extractions with liberal volumes of ether were applied, and the operation conducted in a shaking machine. Each unit maceration with cold ether was continued for about 10 to 12 hours. The percentages of material extracted by the different solvents are recorded in Table I.

Attention is called especially to the difference in proportions of material extracted by ether from autoclaved and unautoclaved cells. Whether this is due to the different temperature conditions



of extraction, to changes brought about in the cells by autoclaving, or by the effect of first extracting the bacteria with alcohol was not decided. The results obtained by analysis of the defatted cells are recorded in Table I.

It is to be noted that the nitrogen and phosphorus values are higher in the case of the autoclaved cells where more thorough extraction was applied to remove fatty and waxy products of the cell.

TABLE I.  
*Analysis of Bacteria.*

		Autoclaved cells.	Unautoclaved cells.
		<i>per cent</i>	<i>per cent</i>
Composition.	Moisture.		2.16
	Nitrogen.	11.05	11.38
	Phosphorus.	1.62	1.34
	Ash.	5.25	3.41
	P <sub>2</sub> O <sub>5</sub> in Ash.	65.50	69.00
Fat extraction.	Alcohol.	1.91	
	Toluene.	2.27	
	Ether.	8.13	2.58
Defatted bacteria.	Nitrogen.	12.31	11.47
	Phosphorus.	1.64	1.25

*Proximate Analysis of Bacterial Cells.*

The scheme of proximate, bacterial analysis, which was applied in our work, was in accordance with the plan described by Johnson and used in the researches on tubercle bacilli. This is recorded in a paper recently published from this laboratory (3). Unless stated otherwise the temperature at which all extractions were applied was 35° for the autoclaved cells and 25° for the unautoclaved.

After the defatting process, all bacteria were again pulverized. 57 gm. of the autoclaved and 80 gm. of the unautoclaved cells were then extracted four times respectively with 200 cc. portions of distilled water for 30 hours per extraction. A few drops of chloroform were added as a disinfectant. These aqueous solutions were then clarified with a low power centrifuge and finally with the

supercentrifuge. After this treatment all the solutions gave good biuret tests, and also Molisch, Hopkins-Cole, and Millon's reactions. Neither solution gave a positive Fehling's test for sugar before digestion with strong hydrochloric acid, while only that from the autoclaved cells gave a sugar test after hydrolysis. De-

TABLE II.  
*Proximate Analysis of Defatted Bacterial Cells.*

		Autoclaved cells (57 gm.).		Unautoclaved cells (80 gm.).	
		<i>per cent of total</i>	<i>per cent</i>	<i>per cent of total</i>	<i>per cent</i>
A. Water extract.	Nitrogen.	10.37		8.41	
	Phosphorus.	24.34		15.35	
B. After dialysis.	Nitrogen.	1.62		3.82	
	Phosphorus.			5.00	
C. Sodium chloride extract.	Nitrogen.	10.06		7.94	
	Phosphorus.	31.19		11.50	
D. After dialysis.	Nitrogen.	7.20		2.52	
	Phosphorus.			5.22	
E. Final residue. Jell mass.		<i>gm.</i>		<i>gm.</i>	
		9.09		14.50	
		1.30		2.62	
F. Final residue.  Jell mass.		<i>per cent of total</i>		<i>per cent of total</i>	
	Nitrogen.	12.97	10.01	16.73	10.59
	Phosphorus.	2.02	0.208	3.13	0.22
	Nitrogen.	2.38	12.09	3.53	12.43
	Phosphorus.	0.36	0.255	0.57	0.41
G. Alkali extracts (0.5 per cent NaOH).	Nitrogen.	35.28		32.80	
	Phosphorus.	22.93		64.17	

terminations of nitrogen and phosphorus in 10 and 25 cc. aliquot fractions of these water extracts respectively gave the values for nitrogen and phosphorus which are recorded in A of Table II. The analytical values recorded are expressed as percentages of the total nitrogen and phosphorus in the dry defatted bacteria.

These aqueous solutions were dialyzed under sterile conditions

for 2 weeks. At the end of that time the biuret and Molisch tests were still positive. The solution from the autoclaved cells gave a reduction test for sugar with Fehling's solution but only after acid hydrolysis. An analysis of these dialyzed solutions gave the values which are recorded in B of Table II.

As is shown by these analytical results the greatest proportion of the extractable nitrogen and phosphorus of the bacteria is in a dialyzable form. These results are thus in accord with those obtained in the research on tubercle bacilli. Upon saturating these two solutions with ammonium sulfate only very slight protein precipitates were obtained. These were dissolved in distilled water and then dialyzed until ammonia-free as shown by Nessler's reagent. To the solutions were then added 2 volumes of alcohol when only a slight opalescence was produced, and the precipitation of protein in both cases was so small that it could not be examined further.

The residues of autoclaved and unautoclaved cells remaining after cold water extraction were given four successive extractions with 200 cc. portions of 5.0 per cent sodium chloride solution, each extraction being applied for 24 hours. The supercentrifuge was used for filtering and clarifying the saline solution. After washing, the combined extracts gave respectively faint biuret and Molisch reactions, but in neither case were tests for sugar with Fehling's solution obtained. Millon's test was also negative, while the solution from the unautoclaved cells gave a very faint Hopkins-Cole reaction. The percentages of nitrogen and phosphorus extracted by the sodium chloride solution are recorded in C of Table II.

The percentages of nitrogen extracted by sodium chloride are almost the same as were removed from the cells by cold water extraction. It is also interesting to note that the percentage of extractable phosphorus compounds is the highest in the case of the autoclaved cells. That a very large proportion of the nitrogen and phosphorus extracted by the sodium chloride solution is in a dialyzable form is revealed by the analytical determinations, the results of which are recorded in D of Table II. The figures recorded represent the percentages of the total nitrogen and phosphorus remaining after dialyzing the respective solutions until free from chlorides. The Molisch test was positive. The amount

of insoluble material remaining in the dialyzing bags was too small for further investigation. It did not respond to a biuret test.

To the dialyzed solutions, free from chlorides, ammonium sulfate was now added to the point of complete saturation. A heavy precipitate deposited in the solution from the autoclaved cells, and only a very slight precipitate in the solution from the unautoclaved cells. The difference in behavior was very characteristic and suggests deep seated changes brought about by the autoclaving of the bacteria. Both precipitates dissolved in cold water and the aqueous solutions were dialyzed until free from ammonia. On adding an equal volume of alcohol to each solution, a heavy colorless precipitate deposited in the solution from the autoclaved cells, while in the other case only a slight opalescence was produced.

The colorless precipitate was separated with the centrifuge, washed repeatedly with alcohol, and finally with ether. After drying in a vacuum over concentrated sulfuric acid it weighed 2.1 gm. This responded to none of the tests for proteins. It was soluble in water and gave no reduction test for sugar when tested with Fehling's solution, even after long hydrolysis with hydrochloric acid. Analysis: ash 26.65, nitrogen 12.48, calcium 6.24, phosphorus 4.68, hydrogen 3.84, carbon 29.74, oxygen 40.47, sulfur 2.55 (per cent). From the evidence that we have thus far obtained it is apparent that we are not dealing here with a definite chemical entity.

The bacterial residues remaining after the brine extraction were treated with 200 cc. of 0.5 per cent sodium hydroxide solution, and the mixture allowed to stand about 48 hours. This treatment was repeated five times in the case of the autoclaved cells. The unautoclaved cells received four treatments of 24 hours each.

All the alkali extracts of the autoclaved cells were combined and finally run through the supercentrifuge. In the case of the unautoclaved cells, the first and second extractions contained the chief proportion of extractable material and were combined and supercentrifuged until clear. The residue was then extracted twice more with 0.5 per cent alkali and finally supercentrifuged again until clear. These residues were finally washed with water and supercentrifuged. The fractions left undissolved by this treatment are called the "final residues." Upon supercentrifuging the wash water twice more in a high power machine clear

amber-colored jells were finally obtained in each case. These are called the "jell masses." Without the use of the Sharples high power supercentrifuge it would have been impossible for us to have separated efficiently these colloidal jell substances.

Both the final residues and the jell masses were washed with 50, 75, 85, and 95 per cent alcohol, twice with absolute alcohol, and twice with cold ether. They were then dried in a vacuum over sulfuric acid. The weights of all these products are recorded in E of Table II.

The final residues gave positive biuret reactions, and also excellent tests for tryptophane and tyrosine. After hydrolysis with hydrochloric acid, sugar was easily detected by reduction of Fehling's and Benedict's solutions. The jell masses gave only weak biuret, tryptophane, and tyrosine tests. On hydrolysis with acids a very strong reduction test with Fehling's solution was obtained. In other words, both of these insoluble fractions contain appreciable amounts of reducing sugars in chemical combination. Regarding their nature we as yet have no knowledge, but it is very probable that we are dealing here with glucoprotein combinations. The results of nitrogen and phosphorus determinations are recorded in F of Table II.

The 0.5 per cent sodium hydroxide extracts and washings were combined. These gave strong biuret, xanthoproteic, Hopkins-Cole, and Millon's tests. After mild hydrolysis with hydrochloric acid sugar combinations were released and were easily detected by reduction of Fehling's solution. The results of nitrogen and phosphorus determinations in the alkaline extracts are recorded in G of Table II.

Proteins were precipitated at once by acidifying these alkaline extracts with acetic acid. They were separated by means of a centrifuge and purified by redissolving in 0.5 per cent sodium hydroxide and precipitating again with acetic acid. This treatment was repeated three times, and the protein fractions were then washed with 50, 75, 85, and 95 per cent alcohol, twice with absolute alcohol, and four times with ether. After drying these products in a vacuum over sulfuric acid we obtained the yields and analytical results recorded in A of Table III.

Neither one of these alkali-soluble proteins from the autoclaved or unautoclaved bacteria gave tests for sugar either before or

TABLE III.  
*Analyses of Alkali-Soluble Proteins.*

Autoclaved cells.				Unautoclaved cells.													
gm.		per cent		gm.		per cent											
9.61		14.51		18.55		12.77											
0.42		0.42				2.30											
Per cent of total N.																	
I	II	I	II	I	II	III	IV	I	II	III	IV						
4.20	3.43					5.04	4.74										
4.75	5.15					11.66	10.05					5.16					
61.78	61.77					50.74	50.13					9.36	8.92				
												54.35	56.34				

after hydrolysis with hydrochloric acid. A positive nitroprusside reaction for sulfur was obtained with both proteins after fusion with sodium. The characteristic color faded, however, very rapidly. The results of nitrogen distribution analyses according to the Van Slyke procedure are recorded in B of Table III.

The mother liquors from which the alkali-soluble proteins had been precipitated were evaporated under diminished pressure to a volume of 200 cc. Upon the addition of hydrochloric acid no precipitate was obtained in the solution from the unautoclaved cells. A heavy precipitate was obtained, however, in the filtrate from the autoclaved organisms. Hydrochloric acid was then added until no further precipitation was produced. This was followed by dilution with an equal volume of 95 per cent alcohol. The solution was cooled overnight and the colorless precipitate then filtered off, washed with 50, 75, and 95 per cent alcohol, and finally with absolute alcohol and ether. It was dried in a vacuum over sulfuric acid. This product weighed 2 gm. It gave a slight biuret test, a strong test for phosphorus, and reduced Fehling's solution after hydrolysis with strong hydrochloric acid. This product was considered to be a part of the nucleic acid fraction and has been set aside for future research.

The effect of autoclaving on the chemical structure of the bacterial cell is very pronounced. In both the autoclaved and unautoclaved bacteria a large amount of the water- and sodium chloride-extractable material is in the form of small molecules capable of penetrating the pores of the dialyzing bag. The presence of materials which give the biuret, tyrosine, and tryptophane tests before dialysis, and the practical disappearance of these tests after dialysis indicate the presence of small peptide molecules and free amino acids. The great decrease in the percentages of total nitrogen and phosphorus further strengthens this conclusion. In other words, autoclaving of the bacteria exerts a great influence upon the rate of hydrolysis or breakdown of the cytoplasmic constituents of the cells. That this is a deep seated change is evidenced by the fact that one obtains a large amount of water-soluble material by the brine extraction of the autoclaved bacteria, and a much less quantity in corresponding extracts of the unautoclaved cells.

Albumin if present in this bacterial cell is in very small quanti-

ties. Its absence cannot be refuted, however, because of the persistence of the biuret test in the water extract, after dialysis, and the obtaining of a very slight precipitate upon saturating the solution with ammonium sulfate. This product was obtained in too small amount from the bacteria at our command for a successful investigation. The presence of a globulin fraction is also very doubtful as the slight precipitate found in the parchment bag after dialyzing out the sodium chloride was not soluble in sodium chloride solution, nor did such a solution give a biuret reaction or other characteristic protein tests.

The sodium hydroxide extractions are also changed in composition by the process of autoclaving as is shown by the results of analyses recorded in G of Table II. The cell change seems to affect the nitrogen distribution less than that of the phosphorus.

The experimental evidence strongly points to these alkali-soluble proteins as being conjugated phosphoproteins and not nucleoproteins. Repeated tests for carbohydrates except by the Molisch test failed to reveal their presence. The Molisch test, as is well known, is very sensitive. All proteins were repeatedly redissolved in dilute alkali, the resulting solutions centrifuged, and then reprecipitated by addition of acetic acid. This treatment should have removed all phosphates which were not combined in the protein molecule. However, the percentage of phosphorus is still high. The protein from the autoclaved cells contains 0.42 per cent, while the protein obtained from the unautoclaved cells contained 2.22 and 2.37 per cent. The comparatively small amount of phosphorus in the protein from the autoclaved cells again reveals the constitutional effect of autoclaving the bacteria.

A study of the nitrogen distribution by the Van Slyke method of analysis reveals further striking differences. The humin nitrogen does not vary to any appreciable extent, being about 4.0 per cent in the protein from the autoclaved and 5.0 per cent in the protein from the unautoclaved bacteria. The greatest difference is to be found in the amide fraction, being approximately 5.0 per cent in the protein of the autoclaved and 10.0 per cent in that from the unautoclaved cells. Another great difference is to be found in the amino fraction, this being about 61.0 per cent in the protein from the autoclaved and approximately 50.0 per cent in the protein from the unautoclaved. It will be noted also that the lysine fractions are quite high.



The examination of the mother liquors, obtained after acidifying and filtering off the alkali-soluble proteins, reveals the probable existence of nucleic acid combinations in these solutions. Both solutions revealed the same content so far as empirical tests showed, yet the solution from the unautoclaved cells did not yield a precipitate that corresponded in its behavior to that of a true nucleic acid. The final residues are apparently the same product in autoclaved and unautoclaved cells and correspondingly the jell masses. They consist of small amounts of protein, traces of nuclear material, and unknown carbohydrates.

It is now possible to conclude from results that have been obtained in the research in this laboratory on tubercle bacillus and from the data presented in this paper that these two organisms differ radically in their cell composition. Not only do they differ in their relative proportions of fats and carbohydrates, but also fundamentally in the character of their protein constituents. Tubercle bacillus contains an appreciable amount of a water-soluble protein of the albumin type while *Bacillus lactis aerogenes* does not. From a chemical standpoint, it is very significant that the water-soluble albumin fraction is found in the pathogenic and not in the non-pathogenic organism. The analytical determinations made for distribution of nitrogen by the Van Slyke method in the alkali-soluble proteins show that each organism, tubercle bacillus and *Bacillus lactis aerogenes*, is apparently characterized by its own specific protein. The protein from tubercle bacillus contains a much higher percentage of arginine and a smaller proportion of lysine than the corresponding protein from *Bacillus lactis aerogenes* (4). The protein of the latter organism is characterized by its high amide nitrogen content.

#### SUMMARY.

1. The bacterium *Bacillus lactis aerogenes* has been grown in quantity on a synthetic medium and subjected to a proximate chemical analysis.

2. A study of the effect of autoclaving the bacteria reveals the fact that this technique causes deep seated changes in the composition of the cell. So radical a change is produced by autoclaving that the products of extraction by the same procedure are not comparable.

3. A study of the nitrogen distribution by the Van Slyke method has been made on the major alkali-soluble protein fraction of the bacterial cell. The analytical results reveal a characteristic protein entirely distinct from the corresponding protein extracted from tubercle bacillus.

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## THE PREPARATION AND PURIFICATION OF LECITHIN.

BY P. A. LEVENE AND IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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A revision of the methods of preparation and purification of lecithin has been undertaken for more than one reason. First, all the older methods employed in other laboratories as well as in our own are costly and time-consuming; second, judging from the numerous and frequent requests for the material which we have had from American and European colleagues, there seems to exist among biologists a considerable need for pure phosphatides.

The mode of purification employed in our laboratory has been continually modified depending upon the source and upon the condition of the material when it arrived at the laboratory. The procedure suggested in the present note is very simple and is applicable to all tissues. By it pure amino-free lecithin can be prepared within 24 to 48 hours.

However, the conditions for the preparation of the crude extracts may vary, depending on the starting material. The preparation of the extracts will be described separately for the three principal sources of lecithin; namely, for egg yolk, for brain, and for liver tissue.

### *Preparation of Crude Cadmium Chloride Salt of the Lecithin Fraction.*

**Egg Yolk.**—Fresh egg yolks are made into a homogeneous emulsion by means of a mechanical stirrer (when such is lacking an ordinary egg beater can be used). The solution is strained through cheese cloth and poured into a double volume of hot 95 per cent alcohol. The alcoholic extract is allowed to cool and to it a cold saturated solution of cadmium chloride in methyl alcohol is added in quantity sufficient to produce a complete precipitation of the lecithin.

*Liver Tissue.*—40 pounds of fresh organs are freed from adhering adipose tissue, minced in a chopping machine, and dried. The dried tissue is extracted with 28 liters of 95 per cent alcohol. The alcoholic extract is concentrated to  $\frac{1}{3}$  the original volume, and the solution is kept overnight in a refrigerator at 0°C. in order to allow the white matter to crystallize. To the filtrate a cold methyl alcoholic solution of cadmium chloride is added in quantity sufficient to complete the precipitation of lecithin.

*Brain Tissue.*—40 pounds of brain tissue are freed from adhering foreign tissue, minced in a chopping machine, and dried in a vacuum drying oven. The material is extracted twice with acetone (16 liters) and the residue is extracted with hot 95 per cent alcohol (24 liters). The further steps in the preparation of the crude cadmium chloride salt are the same as in the preparation of liver lecithins.

#### *Purification of the Cadmium Chloride Salts.*

Two conditions are essential to avoid decomposition of the lecithins in the course of their preparation. The first is to avoid high temperature when effecting solution or concentration and the second is to reduce to a minimum the use of water.

The principal impurity of the crude cadmium salts of lecithins is cephalin. In order to remove this substance the cadmium salts are well shaken with ether and the suspension is centrifugalized. The operation is repeated from eight to ten times. With the exception of the material extracted from liver, which still retains a yellow coloration, the cadmium salts are then perfectly white. At this stage, the cadmium salts contain very small proportions of cephalin. For further purification they are suspended in chloroform (400 cc. of the solvent for 100 gm. of the cadmium salts) and the suspension is shaken at room temperature until a slightly opalescent solution is formed. To this solution a cold 25 per cent solution of ammonia gas in dry methyl alcohol is added as long as a precipitate is formed. It is desirable to avoid a large excess of the reagent. The precipitate is removed by centrifugalization. The precipitate may be extracted with chloroform and the solution treated with methyl alcoholic ammonia.

The combined chloroform-methyl alcohol solutions of the lecithins

thins are concentrated at about 10 to 15 mm. pressure, maintaining the water bath at 35–40°C. To obtain the residue in as dry a state as possible, it is dissolved in dry ether and concentrated to dryness under the same conditions as before. This operation is repeated three times. The final residue is extracted with 99 per cent alcohol. Should an appreciable proportion of cephalins be present in the lecithins, they remain as a residue insoluble in alcohol. Often, however, the residue is completely soluble in alcohol. This solution is treated with a methyl alcoholic cadmium chloride solution and the lecithins are liberated from cadmium chloride as before. The cadmium-free dry residue is dissolved in a minimum volume of ether and poured into acetone. 500 cc. of acetone suffice for the residue obtained from 100 gm. of the cadmium salts.

#### *Further Purification of Lecithins.*

The lecithins prepared in this manner form a light yellow mass and in the case of the brain, a practically white, semisolid mass. The material analyzes correctly for lecithin save for the nitrogen value which is generally slightly higher than 2 per cent. This high nitrogen value is due to small quantities of ammonia. The best procedure to remove this impurity is the following. 50 gm. of lecithin are dissolved in 50 cc. of ether. An equal volume of 10 per cent acetic acid is added and the mixture is shaken in a shaking machine for  $\frac{1}{2}$  to 1 hour. A thick emulsion is formed which is poured into 500 cc. of acetone. The supernatant liquid is decanted and the precipitate is washed repeatedly with dry acetone. The wash acetone is added to the decanted water-acetone solution and the combined solution is concentrated under reduced pressure to dryness. The residue is dissolved in dry ether and the lecithin contained in it is precipitated by means of acetone. As a rule, the total loss of material is very small. The first, the purer material, contains about 50 per cent and the second about 25 per cent of the original lecithin.

#### *Analytical.*

*Egg Lecithin.*—Several samples of lecithin were prepared. The sample reported here had been reprecipitated as a cadmium chloride salt seven times. In this case both the portion precipitated

from the emulsion by means of acetone and the one obtained on concentration of the acetone solution analyzed correctly.

46.0 gm. of the material were dissolved in 50 cc. of ether and emulsified with 50 cc. of water. The emulsion was poured into 500 cc. of acetone. The precipitate contained 26.0 gm. of lecithin; from the mother liquor, on concentration, 10 gm. of lecithin were obtained.

The first 26.0 gm. were again emulsified and the lecithin precipitated with acetone. The yield this time was 22 gm.

*Brain Lecithin.*—The sample was reprecipitated with cadmium chloride six times. It was repurified by the acetic acid emulsion process only once (No. 170). The yield from 20 pounds of the brain tissue was 26 gm. On purification the yield was 19 gm. in the precipitate and 5 gm. were recovered from the mother liquor.

*Liver Lecithin.*—The material was reprecipitated with cadmium chloride three times. The yield was 50 gm. of lecithin from 40 pounds of fresh liver tissue. The material was purified with dilute acetic acid once.

The composition of the samples was as follows:

	C	H	N	$\frac{\text{NH}_4\text{-N}}{\text{Total N}}$	P	Iodine value.
<b>Egg lecithin.</b>						
First fraction (No. 134) .....	65.46	10.54	1.98	0	3.96	70
“ “ repurified (No. 152) .....	66.01	10.59	2.03	0	3.90	62
Second fraction (No. 135) .....	65.63	10.58	2.09	0	3.97	70
<b>Brain lecithin (No. 170) .....</b>	<b>64.83</b>	<b>10.61</b>	<b>1.99</b>	<b>0</b>	<b>3.90</b>	<b>61</b>
“ “ repurified (No. 171) .....	65.53	10.69	2.00	0	3.90	47
<b>Liver lecithin .....</b>	<b>64.83</b>	<b>10.19</b>	<b>2.24</b>	<b>0</b>	<b>3.99</b>	<b>90</b>
“ “ repurified .....	65.45	10.25	2.17	0	3.93	
<b>Calculated.</b>						
Oleic palmitic lecithin .....	64.81	10.81	1.80	0	3.99	32.7
“ stearic “ .....	65.55	11.01	1.73	0	3.85	31.5
Linolenic palmitic lecithin .....						98.1

# CONFIGURATIONAL RELATIONSHIPS OF METHYLETHYL AND METHYLPROPYL CARBINOLS.

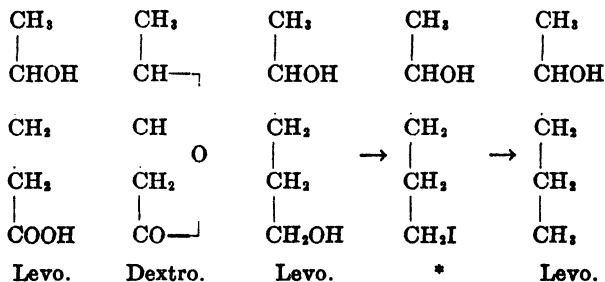
By P. A. LEVENE, H. L. HALLER, AND A. WALTI.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

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The configurational relationship of dextro-methylethyl carbinol to dextro-lactic acid has been established.<sup>1</sup> The establishment of the relationship of the higher members of the homologous series of alcohols to the same reference substance will permit the correlation of the configurations of the alcohols among themselves. The present paper contains a report on the results of the efforts in this direction; namely, the results of experiments leading to conclusions regarding the configurational relationship of levo-methylpropyl carbinol to levo-lactic acid.

The task was facilitated by the fact that the configurational relationship of 4-hydroxyvaleric acid to lactic acid had already been established. Hence, it was necessary only to correlate 4-hydroxyvaleric acid with methylpropyl carbinol. The following set of reactions led to the desired end.



<sup>1</sup> Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 465.

\* Rotation not determined.



## 592 Methyleneethyl and Methylpropyl Carbinols

The methylpropyl carbinol was identified by means of its  $\alpha$ -naphthylurethane. The rotation of the urethane was in the same direction as that of the alcohol. In order to verify this conclusion, a naphthylurethane was prepared from dextro-methylpropyl carbinol obtained by resolution of the inactive carbinol. In the latter case also the carbinol and the urethane rotated in the same direction.

Thus it was found that the configuration of levo-methylpropyl carbinol is related to that of levo-4-hydroxyvaleric acid and hence to levo-lactic acid and, *vice versa*, dextro-methylpropyl carbinol to dextro-lactic acid. Inasmuch as dextro-lactic acid already has been correlated to dextro-methyleneethyl carbinol, it follows that dextro-methyleneethyl carbinol and dextro-methylpropyl carbinol are configurationally related.

In the course of the work with 1,3-dihydroxybutane it was found that on treatment with a halogen acid, the reaction product did not consist entirely of 1-halogen-3-hydroxybutane, but contained also a small proportion of 1-hydroxy-3-halogen butane. These two can be separated by fractional distillation. However, for a complete separation large quantities of starting material are needed. Fortunately, for the purpose of the present investigation, the separation of the two possible iodohydrins is not necessary as only glycols substituted in position (1) lead to optically active carbinols.

At this place mention may be made also concerning the stability of the  $<1, 4>$  ring in the amylenoxide. In the process of reduction of 1-iodo-4-hydroxypentane to methylpropyl carbinol, the oxide with a  $<1, 4>$  ring is formed as a by-product. It was found impossible to hydrolyze the oxide into the corresponding glycol, whereas ethylene or propylene oxide adds on a molecule of water rapidly.

### EXPERIMENTAL.

*Dextro- $\gamma$ -Valerolactone.*—This lactone was obtained from the mother liquors in the resolution of  $\gamma$ -hydroxyvaleric acid with cinchonidine.<sup>2</sup> The solvent was removed by distillation under reduced pressure. The salt was dissolved in water and concen-

<sup>2</sup> Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxi, 165.

trated sulfuric acid was added until the reaction mixture was acid to Congo red. The solution was heated on a water bath for 15 minutes, cooled, filtered, and extracted with ether in a continuous ether extractor. The ethereal extract was dried over anhydrous sodium sulfate and after removal of the solvent, the lactone was distilled. It boiled at 86-90°,  $p = 14$  mm.

*Levo-1,4-Dihydroxypentane*.—Dextro- $\gamma$ -valerolactone ( $\alpha_D^{20} = +13.5^\circ$  without solvent in 1 dm. tube) was reduced in 10 gm. lots with sodium and glacial acetic acid in the apparatus described by Levene and Allen.<sup>3</sup> 13.8 gm. of sodium were emulsified in 100 cc. of dry toluene and 10 gm. of the lactone dissolved in 20 cc. of glacial acetic acid were then introduced. The solution was added at such a rate that the introduction required 8 minutes. During the course of the reaction, 100 cc. of toluene were gradually added through the condenser. After the addition of the lactone, 16 cc. of glacial acetic acid in 20 cc. of toluene were added followed by 25 cc. of absolute alcohol when refluxing had ceased. The reaction mixture was cooled and filtered. The filtrate was concentrated under reduced pressure. To the residue absolute alcohol and ether were added, the precipitate was filtered off, and the filtrate, which was acid to litmus but not to Congo red, was concentrated on the water pump to a thick syrup. This was distilled on a high vacuum pump. The glycol distilled at 95-96°C.,  $p = 1.5$  mm.

0.1404 gm. substance: 0.2974 gm.  $\text{CO}_2$  and 0.1458 gm.  $\text{H}_2\text{O}$ .

$\text{C}_5\text{H}_{12}\text{O}_2$ . Calculated. C 57.70, H 11.54.

Found. " 57.76, " 11.62.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-1.55^\circ \times 100}{1 \times 31.6} = -4.90^\circ.$$

55 gm. of lactone yielded 44 gm. of the glycol. 30 gm. of lactone ( $\alpha_D^{20} = +10.25^\circ$  without solvent in 1 dm. tube) were reduced in the same manner as described above and yielded 24 gm. of glycol. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-1.05^\circ \times 100}{1 \times 28.3} = -3.70^\circ.$$

<sup>3</sup> Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

## 594 Methylethyl and Methylpropyl Carbinols

*Di-(Phenylurethane) of Levo-1,4-Dihydroxypentane.*—1 part of 1,4-dihydroxypentane ( $[\alpha]_D^{20} = -4.90^\circ$ ) and 2.4 parts of phenylisocyanate were heated on a steam bath for 1 hour. The reaction mixture was then allowed to stand overnight. The viscous mass was washed with petroleic ether and crystallization was induced with a stirring rod. After washing with petroleic ether, the urethane was recrystallized from 95 per cent alcohol. It melted at 131–133°C. and analyzed as follows:

0.0997 gm. substance: (Kjeldahl) 5.80 cc. 0.1 N acid.

$C_{13}H_{22}O_4N_2$ . Calculated. N 8.18.

Found. " 8.14.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-0.11^\circ \times 100}{4 \times 3.87} = -0.71^\circ.$$

*1-Iodo-4-Hydroxypentane.*—For the preparation of this substance, the combined glycols obtained in the preceding experiment were employed. Into the glycol slightly more than 1 equivalent of dry hydrogen iodide was passed, the reaction mixture being maintained at 0°C. It was then heated on the water bath for 1 hour, cooled, ice and chloroform added, and the mixture neutralized with sodium carbonate. The chloroform extract was dried over sodium sulfate. After removal of the chloroform the remaining iodohydrin was reduced directly without further purification.

*Levo-Methylpropyl Carbinol.*—The iodohydrin obtained in the foregoing experiment was reduced with hydrogen in the presence of colloidal palladium. The procedure was the same as that described<sup>1</sup> for the reduction of 1-iodo-3-hydroxybutane.

The ether was distilled off, using a fractionating column, and the residue was distilled.

Fraction I. 80–84° 8 gm.  $\alpha = -2.18^\circ$  in a 1 dm. tube.

" II. 85–116° 3 "

" III. 116–120° 4 "  $\alpha = -1.20^\circ$  " " 1 " "

Fraction I consisted chiefly of pentylene oxide.<sup>4</sup> Fraction III had the boiling point of methylpropyl carbinol. It was therefore redistilled and converted into the  $\alpha$ -naphthylurethane.

<sup>4</sup> von Ehrenthal, B. P., *Monatsh. Chem.*, 1903, xxiv, 354.

*α-Naphthylurethane of Methylpropyl Carbinol.*—1 part of the alcohol obtained in the above experiment and 2 parts of *α*-naphthylisocyanate were heated on the steam bath for 15 minutes. The reaction mixture was allowed to stand overnight. It was then washed with petroleic ether and extracted with hot absolute alcohol. To the alcoholic filtrate water was added until an oil separated which readily solidified on cooling. It was recrystallized from dilute alcohol. It melted at 71–73°C. and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 1.93 cc. 0.1 N acid.

$C_{16}H_{19}O_2N$ . Calculated. N 5.44.

Found. " 5.40.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-0.42^\circ \times 100}{2 \times 7.5} = -2.8^\circ.$$

The above results were substantiated by converting dextromethylpropyl carbinol into its *α*-naphthylurethane. The carbinol was obtained by the resolution of inactive methylpropyl carbinol as described by Pickard and Kenyon.<sup>5</sup>

The carbinol without solvent had a rotation of  $\alpha_D^{20} = +7.65^\circ$  in a 1 dm. tube. The *α*-naphthylurethane was prepared in the usual manner. It melted at 88–91°C. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+0.65^\circ \times 100}{1 \times 4.9} = +13.3^\circ.$$

It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.90 cc. 0.1 N acid.

$C_{16}H_{19}O_2N$ . Calculated. N 5.44.

Found. " 5.46.

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<sup>5</sup> Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 64.



## THE FATE OF SUGAR IN THE ANIMAL BODY.

### IV. THE TOLERANCE OF NORMAL AND INSULINIZED RATS FOR INTRAVENOUSLY INJECTED GLUCOSE AND FRUCTOSE.

By CARL F. CORI AND GERTY T. CORI.

(From the State Institute for the Study of Malignant Disease, Buffalo.)

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#### INTRODUCTION.

The experiments reported in previous papers of this series made it desirable to study the carbohydrate metabolism of the rat from as many angles as possible. The starting point for the present investigation was the marked difference in the sugar tolerance of the rat and of other species of animals. Woodyatt, Sansum, and Wilder (1) found on rabbits, dogs, and men that glucose may be infused intravenously at a rate of 0.85 gm. per kilo per hour without causing glycosuria, while at a slightly greater rate of infusion sugar escaped in the urine. The intravenous tolerance limit for fructose, as determined by Woodyatt, was close to 0.15 gm. per kilo per hour. Yet, rats absorb glucose at a rate of 1.78 and fructose at a rate of 0.77 per kilo per

hour without showing glycosuria. There seemed to be two explanations possible. Either the rat had actually a higher sugar tolerance than the species examined by Woodyatt, or, the sugar tolerance when tested by the intestinal route was higher than when tested by the intravenous route. In order to decide which of the two explanations is the correct one, the sugar tolerance of the rats has been tested by the intravenous infusion method.

Another question, that presented itself in connection with previous work, was, to what extent insulin injections increased the tolerance for intravenously injected glucose. In the second paper of this series, the glucose that had been absorbed during 4 hours could be accounted for by determining sugar oxidation and glycogen formation on the same animal. It was intended to repeat these experiments during the intravenous infusion of sugar, in the hope that a large surplus of glucose could be administered as the result of the insulin injections. Since this hope was not fulfilled, the original plan had to be abandoned.

Finally it was thought advisable to study the behavior of fructose during intravenous infusion, before recovery experiments with this sugar were attempted. It is known that fructose can be converted into glucose in the tissues. The argument is that if such a conversion takes place to a large extent, the fate of intravenously injected fructose and glucose should be nearly the same. If, on the other hand, marked differences between these two sugars are detected, this would speak against the conception that a conversion plays an important rôle in the disposal of fructose in the tissues.

Later on the preliminary observations just mentioned were extended in various directions and were supplemented by experiments on mice and rabbits.

## II.

### *Methods.*

The sugars used in this work were Pfanstiehl's c.p. products. The solutions in distilled water were freshly prepared, filtered, and adjusted to a pH of 7.4. For the determination of the sugar concentration Bertrand's method was followed.

The experiments on rats were made under amytal narcosis. 8 mg. per 100 gm. of body weight, injected intraperitoneally, produced a very satisfactory narcosis. Even if the anesthesia was extended over 3 hours, the animals recovered completely in a few days. Whenever it seemed advantageous, the same animal was used for a second experiment. The infusions were made into the femoral vein and in some experiments into a mesenteric vein. The sugar solution was delivered from a burette, graduated in 0.02 cc. The device used to secure a constant, very slow flow of the sugar solution from the burette was that described by Burn and Dale (2). From 2 to 3 cc. of sugar solution were infused in 1 hour. Readings of the burette were taken every 2 minutes. In this way the rate of infusion could be kept constant within 0.02 cc. After 1 hour a small opening was made in the abdominal wall and the bladder emptied by gentle compression. In most instances the infusions were extended for 1 or 2 more hours. Occasionally, after the infusion had been stopped, the urine was collected for 1 more hour in order to see whether a lag in the sugar excretion had occurred. This was not the case, even if the rats showed strong glycosuria in the preceding infusion period.

The urine was treated with Lloyd's reagent in order to remove interfering substances and the sugar was determined by the Hagedorn and Jensen method. When fructose was infused, the quantitative estimations were checked by the Selivanoff reaction. The latter was always strongly positive, when the quantitative determination indicated glycosuria. It has been shown in the first paper of this series (3) that rats fasted for 48 hours excrete 0.3 mg. of reducing substances per 100 gm. of body weight per hour. During the absorption of glucose or fructose the rats excreted between 1 and 2 mg. of reducing substances per 100 gm. per hour, the blood sugars ranging from 0.16 to 0.2 per cent in the case of glucose and from 0.14 to 0.17 per cent in the case of fructose. One of the main purposes of the present investigation was a comparison of the tolerance by the intestinal and the intravenous route. An excretion of more than 2 mg. of reducing substances per 100 gm. of body weight per hour was, therefore, taken as indicating glycosuria.

In the experiments with mice the same technique was followed



as on rats. The animals were anesthetized with amytal, the infusions being made into the tail vein. Mice fasted previously for 17 hours excreted a urine that contained, as an average, 0.7 mg. of reducing substances per 100 gm. of body weight per hour. The rate of absorption of sugar from the intestine was determined in the same way as on rats (3). The sugar solution was introduced into the stomach of the unnarcotized mice with the aid of the cannula described by Behrens (4).

In the experiments with rabbits narcosis was not necessary. The animals were placed in a box with a small opening for the head. One ear was fastened to a board with adhesive tape. After the animals had quieted down completely, the infusions were made into the marginal ear vein. From 15 to 20 cc. of sugar solution were infused in 1 hour. The rate of infusion was kept constant within 0.1 cc. The urine was collected by catheter and the sugar determined by the Bertrand method.

### III.

#### *Experiments with Glucose on Fasting and Non-Fasting Rats.*

The rate of absorption from the intestine had been determined on rats fasted previously for 48 hours. The animals on which the intravenous tolerance was determined, were, therefore, fasted for the same length of time. It has been mentioned that a rate of absorption of 1.78 gm. of glucose per kilo per hour did not lead to sugar excretion in the urine. However, glucose could be infused intravenously at a still higher rate without causing glycosuria. Table I indicates that the intravenous tolerance limit is close to 2.5 gm. per kilo per hour. This result shows that the rat has a 3 times higher glucose tolerance than rabbits, dogs, or men. There is in the rat a safe margin between the maximum rate of sugar absorption and the intravenous tolerance rate. This explains why alimentary glycosuria cannot be produced in the rat. It will be shown in the following section that this does not hold true for other species of animals.

Another point of interest was whether fasting reduced the intravenous tolerance for glucose. In a previous communication (5) the intravenous tolerance limit of twelve non-fasting rats was found to be between 2.2 and 2.5 gm. of glucose per kilo per

hour. These experiments were made in September, 1925. A new series of experiments on non-fasting male rats, which is recorded in Table II, was made in July, 1926. Here the intravenous tolerance was close to 2.5 gm. per kilo per hour. It may be concluded that a fasting period of 48 hours is without influence

TABLE I.

*Glucose Tolerance of Male Rats, Fasted Previously for 48 Hours.*

An excretion of more than 2 mg. of sugar indicates glycosuria. The experiments were made in the winter months.

Body weight.	Glucose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Rate of infusion below or above tolerance.
gm.	gm.	mg.	
128.1	0.22	0.5	Below.
	0.32	4.5	Above.
151.9	0.24	1.6	Below.
142.3	0.25	0.9	"
	0.25	1.2	"
152.6	0.25	0.4	"
	0.27	3.8	Above.
128.1	0.26	2.3	"
	0.27	3.0	"
130.0	0.26	2.5	"
118.2	0.26	3.1	"
	0.27	3.8	"
137.0	0.29	11.2	"
	0.00	1.8	

on the glucose tolerance of rats. This was rather surprising, since fasting in other species of animals seems to reduce the ability to utilize glucose. Indeed, Hofmeister as early as 1890 (6) described a condition in fasting dogs, which he called "starvation diabetes." Recently it has been discovered that starvation is without influence on the glucose utilization of the rat in the

winter months only. When the experiments were repeated in June and July, the intravenous glucose tolerance of fasting rats was found to be markedly diminished. The reason for the different behavior will be discussed in the following paper.

TABLE II.

*Tolerance of Non-Fasting Male Rats for Intravenously Injected Glucose.*

An excretion of more than 2 mg. of sugar per 100 gm. of body weight per hour indicates glycosuria. The experiments were made in July.

Body weight.	Glucose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Rate of infusion below or above tolerance.
gm.	gm.	mg.	
186.6	0.20	1.7	Below.
172.6	0.21	1.4	"
	0.23	1.5	"
	0.27	5.0	Above.
213.3	0.21	1.3	Below.
	0.24	1.9	"
200.1	0.23	1.4	"
	0.24	1.8	"
216.0	0.26	3.3	Above.
	0.30	4.9	"

## IV.

*Experiments on Mice.*

The metabolism of the mouse, calculated per unit of weight, is approximately  $1\frac{1}{2}$  times higher than that of the rat. It seemed of interest to inquire whether there was a relation between the height of the metabolism and the rate of intestinal absorption. One would expect that a higher metabolism calls for a greater rate of absorption of foodstuffs from the intestine. Table III indicates that mice absorb glucose at a rate of 4.6 gm. per kilo per hour or  $2\frac{1}{2}$  times as fast as rats. On the other hand, no such relation seems to exist between the height of the metabolism and the intravenous tolerance. Table IV shows that the intravenous tolerance of mice is the same as that of rats, namely 2.5

gm. of glucose per kilo per hour. It will also be recalled that Woodyatt found the same intravenous tolerance for men, dogs, and rabbits, while their metabolism stands in the relation of 1 : 1.6 : 2.3.

TABLE III.

*Rate of Absorption of Glucose from the Intestine of the Mouse.*

The animals were fasted for 17 hours previously. 0.5 cc. of a 60 per cent glucose solution was fed by stomach tube.

Glucose absorbed per 100 gm. of body weight per hr.	Blood sugar.	Sugar excreted per 100 gm. of body weight per hr.	Length of absorption period.	Remarks.
gm.	mg.	mg.	hrs.	
0.473	352	*	1	Average of 7 mice.
0.452	365	77.3	2	" " 6 "

\* Not determined.

TABLE IV.

*Tolerance of Mice for Intravenously Injected Glucose.*

The animals were fasted for 17 hours previously.

Body weight.	Glucose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Blood sugar.
gm.	gm.	mg.	mg.
21.5	0.21 0.23	2.1 2.2	260
27.2	0.25 0.29	2.8 3.9	294
26.1	0.25 0.30	2.2 6.1	389
23.5	0.38	9.2	428
20.4	0.40	15.2	
24.5	0.40	19.3	
25.4	0.00	1.2	167

Table III shows that during the absorption of glucose the mice excreted considerable amounts of sugar in the urine. This was to be expected, since in mice and probably in other species of animals the rate of absorption of glucose is greater than the in-

travenous tolerance rate. However, the absorption of glucose alone is an unphysiological occurrence. It has been shown previously (7, 8) that glucose in the presence of another sugar or of amino acids is absorbed much slower than it is absorbed alone. After the ingestion of a mixed diet, there is a great variety of substances present in the intestine and, consequently, the rate of absorption of sugar from this mixture will be slower.

## v.

*Tolerance of Rats for Intravenously Injected Fructose.*

Since in the experiments with glucose the intravenous tolerance rate was greater than the rate of absorption from the intestine, there was reason to expect that the same would hold true for fructose. This was, however, not the case. With a rate of absorption of 0.77 gm. of fructose per kilo per hour no sugar escaped in the urine, while the intravenous tolerance limit for an infusion into the femoral vein was only 0.35 gm. per kilo per hour (Table V). A combination of factors is responsible for this result. One factor is a low kidney threshold for fructose. An examination of the blood sugar values in Table V reveals that fructose is excreted in the urine, when the blood sugar has risen to 0.150 per cent. Due to the lack of a suitable method the actual fructose concentration in the blood could not be determined. If one assumes that the glucose concentration remains unaltered,<sup>1</sup> the kidney threshold for fructose would be at a concentration of about 0.05 per cent. If, on the other hand, part of the fructose is converted into glucose, the kidney threshold for fructose would be still lower.

However, the low kidney threshold in itself cannot explain the difference in the tolerance, since one might reasonably expect that the kidney threshold remains the same, whether the tolerance is measured by the intestinal or the intravenous route. It is true that the infused animals were under amytal narcosis, but since the amytal did not seem to influence the kidney threshold for glucose, there is no immediate reason to assume that it has this effect in the case of fructose.

<sup>1</sup> With a method to be described later, glucose and galactose were determined in the same blood sample. During the absorption of galactose, the glucose concentration in the blood did not rise above normal.

Yet the kidney factor cannot be dismissed altogether. It gains significance in conjunction with the important rôle of the

TABLE V.

*Tolerance of Rats for Fructose Infused into the Femoral Vein.*

The animals were fasted for 48 hours previously. An excretion of more than 2 mg. of sugar indicates glycosuria. The experiments were made in the winter months.

Body weight.	Fructose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Blood sugar.	Rate of infusion below or above tolerance.
gm.	gm.	mg.	mg.	
124.1	0.100	27.0		Above.
132.2	0.094	15.6		"
	0.000	1.2		
158.2	0.072	8.1	163	"
136.2	0.060	4.7		"
119.4	0.051	3.3		"
	0.058	4.9		"
106.0	0.051	2.8	152	"
183.2	0.029	1.2		Below.
	0.039	4.6	147	Above.
130.7	0.030	0.3		Below.
	0.040	6.2		Above.
	0.050	7.7		"
126.1	0.030	1.3		Below.
	0.037	2.6		Above.
	0.044	8.3	156	"
120.0	0.031	0.9		Below.
	0.040	2.4		Above.
	0.050	4.4	151	"

liver for the removal of fructose from the blood stream. When sugar is absorbed from the intestine, it passes through the liver before it reaches the general circulation. It has been shown

in the third paper of this series (9) that in 2 hours 34 per cent of the absorbed fructose is retained as liver glycogen. If fructose is infused into the femoral vein, the sugar passes first into the general circulation. The fructose concentration in the blood might, therefore, easily rise above the kidney threshold, before the liver has had a chance to intercept this sugar. The validity

TABLE VI.

*Tolerance of Rats for Fructose Infused into a Mesenteric Vein.*

The animals were fasted for 48 hours previously. The experiments were made in the winter months.

Body weight.	Fructose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Blood sugar.	Rate of infusion below or above tolerance.
gm.	gm.	mg.	mg.	
152.0	0.050	0.3		Below.
	0.069	0.7		"
205.2	0.080	3.4		Above.
	0.113	13.7	157	"
	0.000	2.1		
166.2	0.062	0.8		Below.
	0.091	6.2		Above.
	0.119	11.3		"
113.0	0.060	1.6		Below.
	0.080	7.3	153	Above.
113.2	0.060	0.6		Below.
138.4	0.057	0.7		"
161.6	0.065	0.5		"
	0.084	2.9	156	Above.

of this explanation was tested experimentally by infusing fructose into a mesenteric vein. In this way the sugar passes first through the liver as in the case of the intestinal absorption. Table VI shows that the intravenous tolerance rate by this route is close to 0.7 gm. per kilo per hour or almost as high as the rate of absorption of fructose from the intestine.

The above experiments were made on rats fasted previously for 48 hours. It was found later that non-fasting rats had a higher tolerance for an infusion into the femoral vein than fasting rats. This is illustrated in Table VII, where the tolerance is close to 0.65 gm. per kilo per hour. The fact that a fasting period of 48 hours reduces the tolerance for fructose but not for glucose, constitutes a marked difference in the behavior of these two sugars in the animal body.

TABLE VII.

*Tolerance of Non-Fasting Rats for Fructose Infused into the Femoral Vein*

Body weight.	Fructose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Blood sugar.	Rate of infusion below or above tolerance.
<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
175.0	0.042	0.8	142	Below.
	0.062	1.3		"
	0.072	7.9		Above.
183.4	0.051	0.7	146	Below.
	0.062	0.9		"
	0.073	5.6		Above.
190.5	0.064	1.3	137	Below.
	0.079	18.0		Above.
173.4	0.062	1.5	131	Below.
	0.069	8.5		Above.
180.1	0.064	1.9	148	Below.
	0.082	10.6		Above.
142.2	0.060	1.2	141	Below.
	0.070	6.8		Above.

## VI.

*Experiments with Glucose and Fructose Infused above the  
Tolerance Rate.*

It has been shown in the preceding section that the tolerance of non-fasting rats for intravenously injected fructose is four times lower than for glucose. One reason for this marked difference is undoubtedly the low kidney threshold for fructose.



Whether it is the sole reason or whether a low rate of utilization of fructose in the tissues also plays a rôle, cannot be decided as long as the kidney factor is not eliminated. It was thought that with a rate of infusion above the tolerance, leading to an excretion of large amounts of sugar, the influence of the kidney threshold would be less marked. The experiments were made on full grown non-fasting male rats. Both sugars, beginning alternatively with glucose or fructose, were tested on the same animal with an interval of 1 week between each test. The rate of infusion was 4.5 gm. per kilo per hour in each case. The infusions were extended over 2 hours, the urine being collected 1 hour longer

TABLE VIII.

*Excretion of Sugar in the Urine during an Infusion of Glucose or Fructose above the Tolerance Rate.*

4.5 gm. per kilo of body weight per hour were given in each case. The infusions were extended over 2 hours.

Rat No.	Body weight.	Amount of sugar eliminated in urine.		Amount eliminated as per cent of amount infused.	
		Glucose.	Fructose.	Glucose.	Fructose.
	gm.	gm.	gm.		
1	243	0.43	0.59	19.6	27.4
2	242	0.33	0.25	15.8	11.8
3	245	0.30	0.43	13.4	19.7
4	249	0.64	0.58	29.4	25.5
5	267	0.61		25.6	
		0.57		23.4	

in order to make allowance for an after excretion of sugar. The data are recorded in Table VIII. There is apparently no marked difference in the rate of utilization between glucose and fructose. In two cases more fructose than glucose was eliminated in the urine, while the opposite was true in two other cases. It is of interest that in these experiments an infusion rate above the tolerance reveals such a marked individual variability. This is shown by the fact that the amount of sugar eliminated, as per cent of the amount infused, varies from 13 to 29 per cent in the case of glucose and from 12 to 27 per cent in the case of fructose. This is in contrast to the great constancy of the individual response when sugar is infused at or near the tolerance rate.

## VII.

*Influence of Insulin on the Tolerance for Glucose and Fructose.*

Table IX indicates that large doses of insulin raise the intravenous tolerance of fasting rats from 2.5 to 3.0 gm. of glucose per

TABLE IX.

*Influence of Insulin on the Tolerance of Rats for Intravenously Injected Glucose.*

The animals were fasted for 48 hours previously. The first insulin dose (20 units) was given 30 minutes before the start of the infusion and was then repeated every hour.

Body weight.	Glucose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Rate of infusion below or above tolerance.
<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	
128.1	0.32	4.4	Above.
150.1	0.35	9.5	"
163.6	0.32	10.8	"
	0.00	1.3	
129.7	0.28	0.5	Below.
	0.32	1.0	"
	0.36	6.7	Above.
141.8	0.29	1.2	Below.
	0.32	15.2	Above.
146.0	0.31	7.4	"
130.6	0.30	0.4	Below.
	0.32	2.4	Above.
134.6	0.30	1.5	Below.
	0.32	12.7	Above.

kilo per hour or by 20 per cent. It seemed possible that the influence of insulin was less marked on account of the amytal narcosis to which the animals were subjected. Consequently the same type of experiment was repeated on rabbits, where narcosis was not necessary. The tolerance of normal rabbits was

TABLE X.

*Tolerance of Normal and Insulinized Rabbits for Intravenously  
Injected Glucose.*

The animals were fasted for 24 hours previously. The first insulin dose (40 units) was given 30 minutes before the start of the infusion and was then repeated every hour.

Rabbit No.	Body weight.	Normal animals.		Insulinized animal.	
		Glucose infused per kilo per hr.	Sugar in urine.	Glucose infused per kilo per hour.	Sugar in urine.
	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>gm.</i>	<i>mg.</i>
1	1900	0.8	000	1.2	000
		1.1	140	1.4	22
				1.5	91
2	2700	0.8	000	1.3	000
		0.9	000	1.5	75
		0.9	000	1.7	321
		1.0	143		
3	2100	0.8	000	0.9	000
		0.9	000	1.3	000
		1.0	38	1.4	40
				1.5	67
4	2600	0.9	000	1.1	000
		1.2	263	1.2	000
				1.3	46
				1.4	89
5	2700	0.9	000	1.2	000
		0.9	000	1.2	000
				1.3	000
				1.4	32
				1.5	120
6	2500	0.97	43	1.3	23
		1.1	122	1.4	34
				1.6	137

at a rate of 0.9 gm. per kilo per hour (Table X), which is in agreement with the value established by Woodyatt. The same animals were then used for the insulin experiments. The glucose tolerance

increased from 0.9 to 1.3 gm. per kilo per hour or by 40 per cent. The larger increase in rabbits seems to support the view that amytal depresses the effect of insulin on the tolerance, but it may also be due to a species difference or to the fact that the rabbits were not fasted for a comparable length of time.

TABLE XI.

*Influence of Insulin on the Tolerance of Rats for Intravenously Injected Fructose.*

The animals were fasted for 48 hours previously. The first insulin dose (10 units) was given 30 minutes before the start of the infusion and was then repeated every hour.

Body weight.	Fructose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Rate of infusion below or above tolerance.
<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	
126.3	0.035	0.8	Below.
	0.038	3.1	Above.
	0.045	7.2	"
143.5	0.038	2.4	"
	0.040	5.1	"
115.8	0.036	0.9	Below.
	0.041	2.8	Above.
146.0	0.035	1.9	Below.
	0.041	4.3	Above.
132.7	0.034	1.2	Below.
	0.037	2.2	Above.
	0.041	4.7	"
154.3	0.036	2.3	"
	0.039	3.8	"

The experiments with fructose are recorded in Table XI. The outstanding fact is that insulin injections have no influence on the tolerance for intravenously injected fructose. This constitutes another marked difference in the behavior of fructose and glucose in the animal body.

## VIII.

## DISCUSSION.

The results of this investigation, in so far as the carbohydrate metabolism of the rat is concerned, have been discussed in the preceding sections. It may be pointed out that infusion experiments on rats are very satisfactory from a technical standpoint, even though the animals are under amytal narcosis. The individual response is very constant and there appears to be no lag in the sugar excretion. In Table I, for instance, the maximum variation in the tolerance is only 4 per cent. To what extent the amytal narcosis influenced the carbohydrate tolerance of the rats, could not be decided, because control experiments on un-narcotized animals were not possible. The great uniformity of the results makes it probable that the effect of the amytal is slight. Even if the sugar tolerance without amytal were higher, this would in no way affect our conclusions.

While this paper was in preparation, there appeared an article by Wierzuchowski (10) under the title, "Influence of Insulin on Levulose and Glucose Intravenously Administered." The infusions were made on dogs at a rate of 2.0 gm. per kilo per hour, which is above the tolerance rate. The hourly excretion of the amount of sugar infused was, as an average, 12.1 per cent for glucose and 9.7 per cent for fructose. At the peak of insulin action the excretion of glucose fell to a minimum of 0.5 per cent of the amount infused. This corresponds to an increase in the glucose utilization of 12 per cent. On the other hand, the excretion of fructose remained uninfluenced by insulin. These findings are in harmony with the experiments reported in this paper.

Wierzuchowski draws the tentative conclusion that the disposal of fructose in the body is independent of insulin. This conclusion seems justified as far as the intravenous tolerance is concerned, but it does not indicate that fructose is independent of insulin in other respects. It has been shown in the third paper of this series (9) that insulin almost completely suppresses the glycogen formation in the liver from fructose. Furthermore, recent metabolism experiments have given evidence that insulin injections lead to an increased oxidation of fructose.

The question, whether fructose is converted into glucose before it is metabolized in the tissues, cannot be decided at present. It is hoped that further work will make it possible to solve this problem.

## IX.

## SUMMARY.

1. The intravenous glucose tolerance of non-fasting male rats is at a rate of infusion of 2.5 gm. per kilo of body weight per hour. This value applies to all seasons of the year.

2. From October until May rats fasted previously for 48 hours show the same intravenous tolerance for glucose as rats that were not fasted. In the summer months the intravenous glucose tolerance is markedly diminished. (See the following paper.)

3. The intravenous tolerance limit of mice is close to 2.5 gm. of glucose per kilo per hour. The absorption of glucose from the intestine of the mouse proceeds at a rate of 4.6 gm. per kilo per hour and leads to glycosuria. (In the rat the rate of absorption of glucose from the intestine is lower than the intravenous tolerance rate.)

4. The tolerance of fasting rats for fructose, when infused into the femoral vein, is at a rate of 0.35 gm. per kilo per hour. However, for an infusion into a mesenteric vein, the tolerance limit is at a rate of 0.7 gm. per kilo per hour. In non-fasting rats the tolerance limit is at a rate of 0.65 gm. of fructose per kilo per hour.

5. When glucose or fructose is infused above the tolerance rate (4.5 gm. per kilo of rat), there is no marked difference in the rate of utilization of these two sugars.

6. Large doses of insulin raise the intravenous glucose tolerance of fasting rats from 2.5 to 3.0 gm. per kilo per hour or by 20 per cent.

7. Normal rabbits show an intravenous tolerance rate of 0.9 gm. of glucose per kilo per hour. After insulin treatment the tolerance increases to 1.3 gm. per kilo per hour or by 40 per cent.

8. Insulin is without effect on the tolerance for intravenously injected fructose.

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## THE FATE OF SUGAR IN THE ANIMAL BODY.

### V. A SEASONAL OCCURRENCE OF KETONURIA IN FASTING RATS ACCOMPANIED BY CHANGES IN CARBOHYDRATE METABOLISM.

BY GERTY T. CORI AND CARL F. CORI.

*(From the State Institute for the Study of Malignant Disease, Buffalo.)*

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The presence of ketonuria in fasting rats during the summer months has been discovered accidentally. Metabolism experiments on rats fasted previously for 48 hours have been carried on more or less continuously for a whole year. Among the forty-nine fasting, non-protein respiratory quotients, observed from October until May, only three quotients were below 0.700. The remaining quotients ranged from 0.705 to 0.715, which indicated a complete oxidation of fat. When the experiments were continued in May, the relationship was suddenly reversed. Most of the fasting quotients ranged below 0.700, while only a few exceeded this figure. Urine tests revealed that these rats excreted considerable amounts of acetone bodies and this was confirmed by quantitative estimations. The ketonuria of the fasting rats persisted with great regularity throughout the summer and disappeared again in late September. An attempt was made to produce ketosis in winter by keeping the animals for a time at a high temperature before they were fasted. When the rats were kept for 3 weeks between 26–28°C., no ketonuria developed upon fasting. Therefore, the higher outside temperature during the summer did not seem to play a decisive rôle in the production of ketonuria in fasting rats. Since the housing, food, and care of the rats was the same throughout the year,<sup>1</sup> the appearance

<sup>1</sup>The rats used in this laboratory are kept in a large, well ventilated animal house, which does not get very hot in summer and which, beginning with October, is steam-heated throughout the winter. The rats are fed the same ration throughout the year, consisting of 20 per cent whole



of ketonuria in fasting rats during the summer months was attributed to a seasonal change in fat metabolism. After a 48 hour fast, 90 per cent of the total metabolism of the rat is derived from fat, while the remaining 10 per cent arises from protein. One may assume that the glycerol of the fat molecule and part of the proteins act antiketogenically. Apparently, this minimum of antiketogenic substances suffices for the complete oxidation of fat in the winter months but not in the summer months. The summer ketosis of the rats offered a possibility to investigate whether the disturbance in the fat metabolism was accompanied by changes in carbohydrate metabolism.

TABLE I.

*Excretion of Acetone Bodies by Fasting Rats.*

The urine from the 24th to 48th hour of fasting was collected. The experiments were made in October at a room temperature of 25–28°C.

Weight of rat.	Total acetone bodies, calculated as acetone, per 100 gm. of body weight per 24 hrs.
gm.	mg.
148.7	2.2
126.2	1.8
121.8	1.9
123.7	3.1
124.0	1.1
128.5	1.9
137.4	1.7
136.1	3.0
141.2	1.8
157.4	0.9
Average.....	1.9

*Observations on the Excretion of Acetone Bodies by Rats.*

The urine from the 24th to the 48th hour of fasting was collected and analyzed for total acetone bodies by means of the gravimetric method of Van Slyke (1). Table IV contains data

wheat, 20 per cent ground fish, 20 per cent milk powder, 29 per cent ground yellow corn, 10 per cent lard, and 1 per cent cod liver oil. In addition, some fresh vegetables are fed twice a week and dog biscuits are suspended in each cage.

on the excretion of acetone bodies during the summer. It will be noted that the values, expressed in terms of acetone, range from 5.4 to 6.9 mg. per 100 gm. of body weight per 24 hours. The  $\beta$ -hydroxybutyric acid amounted to 67 per cent of the total acetone bodies. This is a much higher excretion than was observed at a later season of the year. In October, under analogous conditions, only 0.9 to 3.1 mg. were excreted (Table I). Even if the rats were kept for 3 weeks in November at a temperature comparable to the summer heat, the excretion of acetone bodies

TABLE II.

*Excretion of Acetone Bodies by Fasting Rats.*

The animals were kept for 3 weeks in an environmental temperature of 26–28°C. before they were fasted. The experiments were made in November.

Weight of rat.	Total acetone bodies, calculated as acetone, per 100 gm. of body weight per 24 hrs.
gm.	mg.
143.7	2.5
176.5	1.3
176.5	1.1
202.6	0.8
187.6	1.6
174.5	3.1
201.0	1.2
191.5	0.6
209.0	1.6
215.1	1.5
Average.....	1.5

did not rise to the summer level. This is illustrated in Table II where the excretion of total acetone bodies, for the second 24 hours of fasting, ranges from 0.6 to 3.1 mg. per 100 gm. of rat. Apparently, the summer heat alone is not responsible for the ketosis of fasting rats. Since seasonal changes are known to occur in the endocrine organs, further investigations will be made on that basis. Non-fasting rats during the summer excreted only 0.4 mg. of acetone bodies per 100 gm. per 24 hours (Table III).

Quantitative determinations of the excretion of acetone bodies

are now constantly carried out in this laboratory, since they have been made a routine procedure on all fasting rats that are used for metabolism experiments. It was found that when the excretion of acetone bodies from the 24th to the 48th hour of fasting exceeds 4 mg. per 100 gm. of body weight per 24 hours, the respiratory quotient, determined after 48 hours of fasting, falls below 0.700.<sup>2</sup> In the previous year, from October until May, 6 per cent of the fasting respiratory quotients were below 0.700. This indicated that the ketosis in fasting rats was not entirely absent in the cold season of the year. Recent observations confirm this, since from October up to the time of writing,

TABLE III.

*Excretion of Acetone Bodies by Non-Fasting Rats in the Summer Months.*

The analyses were made on 24 hour urine samples.

Weight of rat.	Total acetone bodies,*calculated as acetone, per 100 gm. of body weight per 24 hrs.
gm.	mg.
210.0	0.31
200.1	0.37
211.0	0.48
175.3	0.34
Average.....	0.38

\* Corrected for urine blank. The urine blank amounted to 0.58, 0.38, 0.49, and 0.42 mg. respectively.

ketosis occurred in 10 per cent of the fasting rats. However, in July and August, ketosis was observed in 90 per cent of the fasting rats. It should be noted that rats suffering from a cold or from a middle ear infection (McCordock and Congdon (2)), have to be ruled out carefully, since they are liable to show strong

<sup>2</sup> According to Magnus-Levi a maximum of 36 gm. of  $\beta$ -hydroxybutyric acid can be formed from 100 gm. of fat. This would reduce the respiratory quotient for fat from 0.707 to 0.669. Theoretically, the respiratory quotients should be between 0.696 and 0.715, depending on the neutralization of the acid with ammonia or with sodium bicarbonate. Obviously, the amount of  $\beta$ -hydroxybutyric acid excreted by the rats is too small to explain a reduction of the respiratory quotient below 0.700. The reason for this discrepancy will be investigated further.

ketosis upon fasting. The observations recorded in this section are not regarded as completed until a second period of summer ketosis has been studied.

*Intravenous Glucose Tolerance of Fasting Rats during the Summer Months.*

It has been shown in Table I of the preceding paper (3) that from October until May the intravenous tolerance limit of rats fasted previously for 48 hours is at a rate of 2.5 gm. glucose per kilo per hour. When these experiments were repeated in summer on rats with ketosis, it was found that the intravenous tolerance was markedly diminished. This is illustrated in Table IV. The tolerance was only 1.6 gm. per kilo per hour or 36 per cent lower than in the winter months. Whether the ketosis in itself was the direct cause for the lower glucose tolerance, has not been decided. Conceivably, the incomplete oxidation of fat and the lessened ability to dispose of injected sugar might be parallel phenomena of the same metabolic disturbance. It should be emphasized in this connection that the glucose tolerance of non-fasting rats was not diminished during the summer months. (See Table II of the preceding paper (3).)

*Fate of Ingested Glucose during the Summer Months.*

In order to decide whether a decrease in the sugar oxidation or in the glycogen formation was responsible for the lowered glucose tolerance of fasting rats during the summer months, recovery experiments, as described in the second paper of this series (4), were made. Table V indicates that from 721 mg. of glucose absorbed during 4 hours, 131 mg. were oxidized, while 380 mg. were converted into glycogen. This corresponds to a recovery of the absorbed sugar of 71 per cent. In the experiments made in winter, where 750 mg. of glucose were absorbed, the sugar oxidation and glycogen formation amounted to 281 and 388 mg. respectively. Here the recovery of the absorbed sugar was 89 per cent. It will be noted that the amount of glycogen formed was the same in both series of experiments. However, there is a striking difference in the amount of glucose oxidized. The rats suffering from summer ketosis oxidized 53 per cent less glu-

TABLE IV.

*Glucose Tolerance and Excretion of Acetone Bodies of Male Rats Fasted Previously for 48 Hours.*

A sugar excretion of more than 2 mg. per 100 gm. of body weight per hour indicates glycosuria. The experiments were made in July.

Body weight.	Glucose infused per 100 gm. of body weight per hr.	Sugar excreted per 100 gm. of body weight per hr.	Rate of infusion below or above tolerance.	Total acetone bodies, calcu- lated as acetone.*
gm.	gm.	mg.		mg.
143.3	0.24	12.0	Above.	6.3
160.2	0.20	2.7	"	5.4
	0.22	30.2	"	
165.5	0.14	0.5	Below.	Lost.
	0.17	8.3	Above.	
	0.19	24.8	"	
174.5	0.16	0.9	Below.	6.1†
	0.18	26.3	Above.	
205.4	0.15	0.8	Below.	6.9‡
	0.17	18.3	Above.	
	0.18	62.7	"	
142.6	0.15	1.1	Below.	5.6
	0.18	25.7	Above.	
182.4	0.16	0.9	Below.	5.7†
	0.17	5.6	Above.	
194.7	0.16	3.3	"	6.8‡
	0.17	19.7	"	
182.5	0.16	3.4	"	6.6‡

\* Acetone bodies excreted per 100 gm. of body weight in the 24 hours preceding the experiments.

† Urine blank 0.25 and 0.3 mg. respectively.

‡  $\beta$ -Hydroxybutyric acid 68, 70, and 63 per cent of total respectively.

case than the rats that were free from ketosis, though the amount of sugar absorbed was nearly the same in both groups of animals. This decreased capacity to oxidize glucose seems to explain why

the intravenous glucose tolerance of fasting rats was found to be lowered in the summer.

The fact that the glycogen formation was the same in spite of the lower glucose oxidation, is difficult to reconcile with the view that glucose oxidation and glycogen formation are linked processes. One would have to assume that the efficiency of the linked process is greater in summer than in winter, since in the former case 2.9 mols of glucose are converted into glycogen per mol of glucose oxidized, while in the latter case the relation be-

TABLE V.

*Recovery Experiments on Fasting Rats in the Summer Months.*

All values are per 100 gm. of body weight per 4 hours.

Fore period.			Glucose absorption period.								
O <sub>2</sub> *	CO <sub>2</sub>	R.Q.	O <sub>2</sub> *	CO <sub>2</sub>	R.Q.	Glucose absorbed.	Glucose oxidized.	Glycogen formed.	Glucose recovered.	Blood sugar.	Urine N.
gm.	gm.		gm.	gm.		gm.	gm.	gm.	per cent	mg.	mg.
0.718	0.685	0.690	0.727	0.790	0.785	0.745	0.151	0.377	70.9	179	12.88
0.743	0.701	0.682	0.678	0.741	0.790	0.741	0.152	0.364	69.5	202	12.08
0.810	0.756	0.676	0.799	0.842	0.762	0.694	0.110	0.401	73.6	229	12.16
0.754	0.721	0.691	0.684	0.730	0.772	0.706	0.111	0.381	69.7	176	12.34
0.756	0.716	0.685	0.722	0.776	0.777	0.721	0.131	0.380	70.9	194	12.36

\* The average value of the direct and indirect O<sub>2</sub> determination is recorded. The average error between the two determinations was  $1.18 \pm 0.8$  per cent. The difference in the R.Q., when calculated from the direct and indirect O<sub>2</sub> values, was, as an average,  $0.008 \pm 0.005$ .

tween the two processes is only 1.38:1. But it is not certain that the synthesis of glycogen from glucose requires as its motor specifically the energy set free by the oxidation of glucose, a point that has already been discussed in the second paper of this series (4). There is nothing, at present, against the possibility that oxidation of fat or protein may serve the same purpose.

In Table V only 71 per cent of the absorbed sugar could be accounted for by oxidation plus glycogen formation, while in the experiments made in winter 89 per cent of the absorbed sugar

was accounted for. The difference in the recovery corresponds to a loss of 132 mg. of glucose. This loss is far too great to be explained by the errors involved in the different determinations, nor is the loss due to sugar excretion, since the urine was analyzed in each case. One might think that the 132 mg. of glucose were retained as such in the tissues, since the blood sugar values were somewhat higher in the experiments made in summer than in winter (0.194 per cent in summer against 0.176 per cent in winter). However, this difference seems too small to be of significance in

TABLE VI.  
*Seasonal Changes in the Metabolism of Fasting Rats.*

	Oct. until May.	May until Oct.
Total acetone bodies, calculated as acetone, per 100 gm. of body weight per 24 hrs.....	1.9 mg.	6.1 mg.
Intravenous glucose tolerance per kilo per hr.....	2.5 gm.	1.6 gm.
Fasting r.q.....	0.713	0.685
r.q. during 4 hrs. of glucose absorption...	0.838	0.777
Glucose absorbed, per 100 gm. per 4 hrs..	0.750 gm.	0.721 gm.
"    oxidized, " 100 " " 4 " ..	0.281 "	0.131 "
Glycogen formed, " 100 " " 4 " ..	0.388 "	0.380 "
Glucose recovered, " 100 " " 4 " ..	89.2 per cent	70.9 per cent
Urine N, per 100 gm. per 4 hrs.....	12.15 mg.	12.36 mg.
Calories from protein, per 100 gm. per 4 hrs.	0.30	0.31
"    " fat, " 100 " " 4 " ..	1.11	1.57
"    " glucose, " 100 " " 4 " ..	1.05	0.49
Total calories, per 100 gm. per 4 hours....	2.46	2.37
Blood sugar.....	0.176 gm.	0.194 gm.

accounting for the lost glucose. When the rats suffering from summer ketosis received glucose plus insulin, the recovery of the absorbed sugar reached 90 per cent. The better recovery was due to an increase in the sugar oxidation. This result suggests that the insulin production of these rats was insufficient. Staub (5) has shown that an increase in the blood sugar concentration stimulates the pancreas to insulin production. Apparently, the pancreas of rats suffering from ketosis does not respond quickly and accurately to this physiological stimulus and consequently

the amount of insulin available for glucose oxidation is too low. In the experiments made in winter the recovery of the absorbed sugar was close to 90 per cent, whether or not insulin was injected. In summer the recovery was satisfactory only when the hormone production of the pancreas was aided by an insulin injection. The main fact is that ketosis leads to a decrease in the glucose oxidation and that in consequence of this a considerable part of the absorbed sugar cannot be accounted for. The mechanism by which part of the absorbed glucose disappears without being either excreted, converted into glycogen, or oxidized, is difficult to explain and will be investigated further. There is hope that the solution of this problem will give some information about the mechanism of sugar oxidation.

#### DISCUSSION.

The seasonal changes in the metabolism of the rat are summarized in Table VI. It will be noted that the animals suffering from ketosis showed a diminished capacity to oxidize glucose and a decrease in the glucose tolerance, while the amount of glycogen formed in the whole body was not changed.

The following data on the excretion of acetone bodies by rats were found in the literature. Smith and Levine (6) showed that the quantity of acetone bodies excreted by the normal rat is independent of the nature of the diet. On a diet composed largely of fat, protein, or carbohydrate or consisting of a balanced mixture of these three food substances, the excretion of acetone bodies varied from 1 to 3 mg. per day. Fasting produced no appreciable ketosis in the rat. The length of the fast and the time of the year at which these experiments were made are not stated. Wigglesworth (7) found in normal rat urine 0.8 to 2.5 mg. of  $\beta$ -hydroxybutyric acid per day. On a diet consisting of 100 per cent butter fat plus salt a rise in the excretion of acetone bodies was observed.

Southwood (8) produced ketosis in men by a diet poor in carbohydrate and rich in fat; then, after the ingestion of 50 gm. of glucose, sugar was excreted in the urine, while normally there was no glycosuria. The blood sugar curve during ketosis rose higher than normal and showed a greatly delayed return. However, when 3 clinical units of insulin were injected, the blood



sugar curve was normal. Robertson (9), commenting on this work, assumes that the functional activity of the pancreas is reduced during ketosis. Greenwald, Gross, and Samet (10) also found a lower glucose tolerance on a diet deficient in carbohydrates and cite further literature to this effect. Haldane, Wigglesworth, and Woodrow (11) demonstrated a decreased glucose tolerance in men, in which ketosis had been produced by the ingestion of sodium bicarbonate. Goldblatt (12) studied the respiratory metabolism of men suffering from starvation ketosis. 1 hour after the ingestion of 50 gm. of glucose, the respiratory quotient was only 0.75, while normally the quotient reached 0.9. This author also makes the comment that ketosis of fasting men appears more rapidly in warm weather.

There seems to be general agreement that ketosis interferes with the normal utilization of carbohydrates in the body. Our results and those of Goldblatt indicate that the chief disturbance is in the sugar-burning mechanism. Insulin injections correct this disturbance and bring the carbohydrate metabolism back to normal.

#### SUMMARY AND CONCLUSIONS.

1. Rats during the summer months excrete from the 24th to the 48th hour of fasting, an average of 6.2 mg. of total acetone bodies per 100 gm. of body weight per 24 hours. During the winter months the excretion of acetone bodies was only 1.9 mg. per 100 gm. per 24 hours.

2. When rats were kept for 3 weeks in winter at a room temperature comparable to that of summer, the excretion of acetone bodies did not rise, showing that the summer heat is not directly responsible for the occurrence of ketosis in fasting rats.

3. The intravenous glucose tolerance of rats suffering from summer ketosis was at a rate of 1.6 gm. per kilo per hour. This is 36 per cent lower than the values obtained in winter on rats without ketosis (see the preceding paper).

4. Recovery experiments showed that the lower glucose tolerance was due to a lessened ability of the tissues to oxidize glucose, while the glycogen storage in the tissues was not changed. From 721 mg. of glucose that were absorbed in 4 hours by the rats in summer, 131 mg. were oxidized, while 380 mg. were converted

into glycogen. This corresponds to a recovery of the absorbed sugar of 70.9 per cent. In winter, for an almost equal glucose absorption, the oxidation and glycogen formation amounted to 281 and 388 mg. respectively; *i.e.*, the recovery was 89.2 per cent.

5. Insulin injected into rats suffering from summer ketosis increased the amount of glucose oxidized and led to a better recovery of the absorbed sugar.

6. It is concluded that ketosis is associated with a reduced functional activity of the pancreas and in consequence of this, with a reduced capacity of the tissues to oxidize glucose.

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### ACETYL MONOSES. III. ON $\alpha$ -MANNOSE PENTACETATE.\*

By P. A. LEVENE AND ISAAC BENCOWITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, January 21, 1927.)

The ring structure of free monosaccharides and of their derivatives has been the subject of considerable disagreement. It naturally follows that either the postulates of individual authors are not always correct or that the substances discussed by them are not always correct. The purity or homogeneity of the derivatives of mannose particularly has been questioned. Regarding the so called  $\alpha$ -pentacetate of mannose, Hudson<sup>1</sup> writes as follows: "For the present the acetate of + 55 rotation will be left unclassified; the determination of its ring form and even the question whether it may not be a mixture of substances remain outstanding problems." It is, however, peculiar that the values for  $[M]_{D\alpha} - [M]_{D\beta}$  for the two known pentacetates of mannose are the same as for the two common forms of pentacetates of galactose and on this basis Levene and Sobotka<sup>2</sup> are in-

\* The first two papers of the series on monose pentacetates contained several errors, which were kindly called to our attention by Dr. C. S. Hudson.

Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, p. 766, line 16 should read "have been prepared by Dale from methylmannoside and from non-crystalline bromotetracetylmannose." From the crystalline bromo derivative the  $\gamma$ -form of Dale was prepared by us.

On p. 768 line 8 from the bottom should read, "causes a change from levo- to dextrorotation."

On p. 774 foot-note 4, instead of "acetobromomannose" should read "acetochloromannose."

Also, it is regretted that an article by Dr. C. S. Hudson and J. K. Dale in which mention is made of two crystalline methyltetracetylgalactoses had been overlooked by us. It is unfortunate that these two substances were never described in detail and therefore not recorded in *Chemical Abstracts*.

<sup>1</sup> Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1433.

<sup>2</sup> Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, 759, 771.

clined to attribute to these four pentacetates the  $\langle 1, 5 \rangle$  ring structure. It is realized, however, that the pentacetate of  $+55^\circ$  rotation has hitherto been crystallized with great difficulty and was never recrystallized with sufficient rigor and that therefore the skepticism of Hudson regarding its purity is justified.

The method of preparation of the mannose pentacetate has now been improved and it has been found possible to recrystallize the substance nine times without changing its optical rotation or its melting point. The latter, however, has been raised from  $64^\circ$  to  $75^\circ\text{C.}$  and after reaching that point, it remained constant. Thus, there seems to be little doubt as to the purity of the mannose pentacetate of  $+55^\circ$ .

Inasmuch as the explanation of the irregular optical behavior of mannose and its derivatives is of great importance for the further development of the work on the relationships of structure and optical rotation in the sugars, it was concluded to test the optical dispersion of  $\alpha$ -mannose pentacetate.

The rotatory dispersion of homogeneous substances generally exhibits a normal course. Biot classified as normal such dispersions as have the locus of the specific rotations in monochromatic lights on a straight line. He regarded as abnormal the dispersion in which the curve connecting the specific or molecular rotations as a function of the wave length has a maximum. Drude later gave mathematical expressions for the dispersion curves of different substances. In certain cases the molecular dispersion could be represented by the expression

$$[M] = \frac{K}{\lambda^2 - \lambda_0^2}$$

In more complicated cases the course is given by the expression

$$[M] = \frac{K_1}{\lambda^2 - \lambda_1^2} + \frac{K_2}{\lambda^2 - \lambda_2^2} + \frac{K_3}{\lambda^2}$$

Lowry referred to the first form of rotatory dispersion as "simple" and to the latter as "complex."

Biot recognized that the most common cause of irregular dispersion is the non-homogeneous composition of the material of which the dispersion is measured. Hence, there is always a

doubt in regard to the homogeneous character of a substance if it exhibits an irregular or complex dispersion. On the other hand, a simple dispersion speaks in a measure in favor of homogeneity.

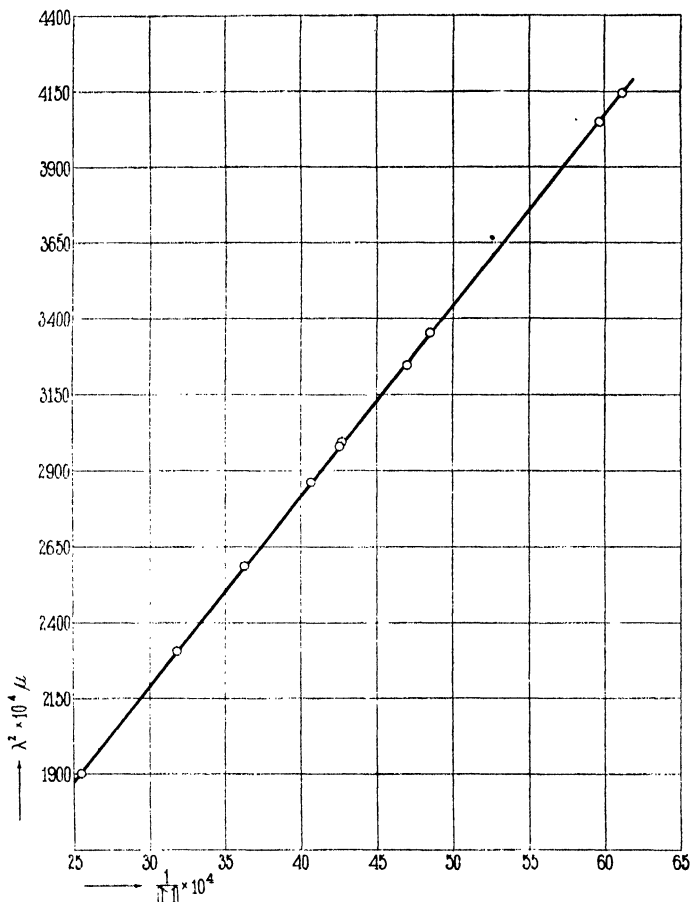


FIG. 1. Optical rotatory dispersion of  $\alpha$ -mannose pentacetate.

The results of the observations on  $\alpha$ -mannose pentacetate are plotted in Fig. 1, where the abscissas represent the reciprocal of the molecular rotations and the ordinates represent the squares of the wave-lengths. It is seen that the experimental points

lie on a straight line. This establishes the accuracy of the experimental technique and indicates that the dispersion of  $\alpha$ -mannose pentacetate can be expressed by one term of Drude's equation. This fact is more strikingly shown in Table I where in Columns 6 and 7 are given the observed and calculated molecular rotations respectively. The calculated molecular rotations were obtained by means of the equation:

TABLE I.

*Optical Rotations of  $\alpha$ -Pentacetyl Mannose for Ten Wave-Lengths.*

$c = 1.174$  mols per 1000 cc.

$\lambda_0^2 = 0.030$

$t = 25^\circ \pm 0.1^\circ$

$K = 7985 \pm 10$

(1)	$\lambda$ (2)	$\alpha$ (3)	Average deviation. (4)	$[\alpha]_{\lambda}^{25}$ (5)	[M] ob- served. (6)	[M] cal- culated. (7)	Differ- ence (8)
			<i>per cent</i>				<i>per cent</i>
Cd red.....	6438	19.19	0.08	41.90	163.3 <sub>9</sub>	163.5 <sub>8</sub>	-0.12
Zn " .....	6364	19.67	0.10	42.94	167.4 <sub>8</sub>	167.7 <sub>4</sub>	-0.15
Cu yellow...	5790	24.16	0.06	52.75	205.7 <sub>1</sub>	206.0 <sub>1</sub>	-0.15
Hg " ....	5700	24.97	0.08	54.51	212.6 <sub>1</sub>	213.2 <sub>6</sub>	-0.30
Ag green....	5472	27.44	0.07	59.91	233.6 <sub>4</sub>	233.4 <sub>6</sub>	+0.08
Hg " .....	5461	27.60	0.06	60.26	235.0 <sub>0</sub>	234.5 <sub>0</sub>	+0.21
Tl " .....	5351	28.86	0.07	63.01	245.7 <sub>3</sub>	245.4 <sub>1</sub>	+0.15
Cd " .....	5086	32.36	0.07	70.65	275.5 <sub>3</sub>	275.0 <sub>6</sub>	+0.17
Cd blue.....	4800	36.85	0.09	80.45	313.7 <sub>6</sub>	313.8 <sub>0</sub>	-0.01
Hg violet...	4359	46.08	0.20	100.6	392.5 <sub>3</sub>	393.0 <sub>0</sub>	-0.12

where [M] is the molecular rotation,  $\lambda^2$  is the square of the wave-length and K and  $\lambda_0^2$  are constants. The differences of the two values given in Column 8 are not only within experimental error but are irregular in magnitude and sign, which establishes the fact that one term of Drude's more general equation:

is sufficient to reproduce the experimental dispersion curve (within our range of wave-lengths and within the experimental precision).

In Column 3 are given the average rotations, in Column 4 are the average deviations of the mean of ten readings.

<sup>3</sup> Levene, P. A., *J. Biol. Chem.*, 1924, lix, 141.



the proportion of the  $\beta$  form is already small, the second fraction may consist in the main of the so called  $\alpha$  form. Such crystallizations have been carried out many times. In the experiment to be recorded here, the second crystalline deposit consisted in the main of the  $\alpha$  form. Its rotation was  $+48^\circ$  and it melted (not sharply) at  $58-60^\circ\text{C}$ . This substance was recrystallized at first out of 40 per cent methyl alcohol, subsequently out of 30 per cent methyl alcohol, and finally out of 25 per cent methyl alcohol. The first crystallizations proceeded very slowly so that between 24 and 48 hours were required for completion of the crystallization. The final crystallizations were completed so rapidly that several recrystallizations could be made within 1 day.

Table II contains the melting points and the rotations of the substances obtained by successive recrystallizations.

#### *B. Procedure for Measuring Rotatory Dispersion.*

All measurements were made with a triple field Schmidt and Haensch polarimeter provided with a large direct vision spectroscope.

The rotations of the solutions were determined in jacketed tubes. A rapid flow of water at  $25^\circ\text{C}$ . maintained a constant temperature. Thermometers divided into tenths of a degree were placed at the inlet and outlet of the tubes. At no time during a run was there a variation of temperature of more than  $\pm 0.1^\circ\text{C}$ .

*Sources of Light.*—The mercury green,  $5461\text{ \AA}$ , and the violet,  $4358\text{ \AA}$ , lines were obtained from a mercury arc. The light of this lamp was purified by the spectroscope and was tested by means of a quartz test plate which was recently calibrated at the Bureau of Standards. This test served as a check on the purity of the light source; *i.e.*, the efficiency of the spectroscope, the effect of possible stray light, and the accuracy of the polarimeter. In every case the rotation of the respective lights by the quartz plate checked the rotations obtained by the Bureau of Standards within the precision limit of our instrument ( $\pm 0.02^\circ$ ).

The Cd, Zn, Cu, Ag, and Tl lines were obtained in the following manner. The light of a small incandescent bulb with a hori-

zontal tungsten filament was focused upon the collimator slit of the spectroscope. The light after having passed the prism, the slit at the telescope end, and the polarizing Nicol prism was rotated by means of the quartz test plate.

The theoretical rotations of our test plate for ten monochromatic lights were calculated. The data of Lowry<sup>4</sup> for the rotation of light in quartz were used. The prism of the spectroscope was then adjusted until the patch of light which passed through the polarimeter was rotated the proper amount. The wave-length of the optical center of this patch was assumed to be the same as that of the monochromatic light which gave a corresponding rotation in the quartz test plate.

TABLE III.

*Comparison of Rotations of the Green Mercury Line 5461 Obtained from the Mercury Arc and That Obtained from a Continuous Spectrum by Means of a Quartz Test Plate.*

Concentration moles per 1000 cc. (1)	$\alpha$ 5461 arc. (2)	$\alpha$ 5461 continuous. (3)	Difference. (4)
			<i>degrees</i>
0.1987	10.20	10.18	0.02
0.4016	10.32	10.29	0.03
0.6124	15.30	15.30	0.00
1.174	27.63	27.60	0.03
2.060	47.16	47.18	0.02

To test the validity of this assumption, especially when the light was rotated by a solution which had a different dispersion curve than that of quartz, the following simple experiment was carried out. Green light from the arc, tested by means of the quartz plate, was passed through the polarimeter and was rotated by several solutions of various strengths. Then the arc was replaced by the incandescent lamp and the spectroscope adjusted until the patch of light illuminating the polarimeter gave the same rotation in the quartz test plate as the light from the arc. The test plate was then replaced by the various solutions and the rotations obtained with this light from the continuous spectrum

<sup>4</sup> Lowry, T. M., *Tr. Phil. Soc.*, 1912, cexii, 261.

of the incandescent lamp were compared with the rotations obtained with the monochromatic light from the arc. The results are given in Table III. The rotations of the green light from the arc are given in Column 2; those of the patch of light from the continuous spectrum are given in Column 3. The differences between the two are given in Column 4. It is seen from these figures that the agreement is remarkable. Additional corroboration of the validity of our assumption is obtained from the results shown in Fig. 1, and given in Table III.

*Manipulation.*—The zero point of the tube filled with the solvent was taken for each wave-length. The tube was then emptied, dried, and refilled with the solution. Before each reading the spectroscope was set by means of the quartz test plate so that the optical center of the patch illuminating the polarimeter had the desired wave-length. At least ten readings for each wave-length were taken. At the end of the run, the spectroscope was readjusted for the various wave-lengths and five additional readings for each wave-length were taken. This served as a check on the setting of the monochromator and indicated any change in concentration which might have taken place during the run.

## ON THE ACTION OF MERCURIC CHLORIDE AND OF HYDROGEN DIOXIDE ON BILE PIGMENTS.

By W. F. VON OETTINGEN AND TORALD SOLLMANN.

*(From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland.)*

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A. Schmidt in 1895 advocated mercuric chloride as a test for the detection of bilirubin and stercobilin in the feces. This was based on the observation that when samples of feces containing bilirubin or stercobilin are triturated with a saturated solution of mercuric chloride and kept for 24 hours at room temperature, the bilirubin sample turns green and the stercobilin sample red. Oppenheimer states in his manual that the turning green of bilirubin feces is due to the formation of biliverdin. This does not necessarily follow, for it is known that green pigments other than biliverdin may be formed from bilirubin, even by oxidizing agents such as halogen elements, which are said to produce green substitution products (Thudichum, Maly). We came to doubt the correctness of this statement as to the bilirubin-mercuric chloride reaction, when working on the action of calomel on the bile; for we noticed that the yellow-green color of the calomel bile which is due to the formation of biliverdin by the oxygen of the air differs distinctly from the blue-green color produced by mercuric chloride. The addition of acid deepens the green color of the biliverdin of the calomel bile, but nevertheless it lacks completely the blue-green tint of the mercuric chloride bile. Furthermore it may be noticed that the formation of the green color in calomel bile starts from the air surface of the test-tube and descends gradually through the liquid. On the other hand, if the bile is poured on the top of a mercuric chloride solution, the blue-green color starts from the plane of contact, and spreads upward with the diffusion of the mercuric chloride. If the two solutions are mixed the color change occurs immediately, while

with calomel the change to biliverdin requires considerable time. The site and speed of the reaction indicate that the color change in the case of the calomel is produced by the atmospheric oxygen; but with the mercuric chloride it is produced directly by the reagent, which presumably furnishes the oxygen by being reduced from the mercuric to the mercurous form. This assumption could be tested by comparing the behavior toward mercuric chloride with that toward other metallic salts of different oxidative potency. It appeared advisable to work with a less complex medium than natural bile, and we therefore prepared some of Küster's "raw bilirubin" from cattle gall stones. The limited supply of this material precluded further purification; nor would it have offered any material advantage. From 25 gm. of powdered gall stones we obtained 2.8 gm. of this "raw bilirubin," as a brown powder, insoluble in water and ether, and only slightly soluble in alcohol and chloroform. With alkalis and alkaline alcohol it went completely into solution, the alkaline solution having a dark brown color which turned greenish on standing in the air. The addition of acid to the alkaline alcoholic solution precipitates nearly the whole of the bilirubin, and the supernatant liquid veers somewhat toward green; alkalization restores the brown color.

*Oxidizing Action of Metallic Salts on Bilirubin.*—The effect of the metallic salts was observed by dissolving the molecular weight of the metallic salt, in mg., in a mixture of 1 cc. of water and 1 cc. of concentrated hydrochloric acid (sp. gr., 1.19), and mixing with 2 cc. of the alkaline alcoholic bilirubin solution. This gave a high concentration of the free acid which made it unnecessary to take account of differences in the hydrolytic dissociation of the salts. The proper color of  $\text{FeCl}_3$ ,  $\text{MnSO}_4$ , and  $\text{Co}(\text{NO}_3)_2$  caused so much interference that these salts could not be utilized. The color of the  $\text{CuSO}_4$  and of  $\text{Ni}(\text{NO}_3)_2$  was also disturbing, but could be discounted by comparing the mixed solution with the combined color of the separate solutions, placed in test-tubes behind each other.  $\text{HgCl}_2$ ,  $\text{ZnSO}_4$ , and  $\text{CdSO}_4$  presented no complication. A control experiment was made by adding the acid without any metal to the bilirubin solution, in the same proportion. This gave a yellow-green color, which underwent no further change on standing 2 hours.

In their effects on this color, the metals can be arranged into

three groups: (1)  $\text{HgCl}_2$  changes the color to a dark green with a blue tinge, which can be pushed further toward blue by heating. (2)  $\text{CuSO}_4$  and  $\text{Ni}(\text{NO}_3)_2$  change the bile pigment to dark green, which cannot be pushed toward the blue by heating. (3)  $\text{ZnSO}_4$  and  $\text{CdSO}_4$  produce no change even on heating. These colors are not materially changed by standing.

The results of these experiments show that other oxidizing metallic salts affect the bile pigment similarly to mercuric chloride although apparently not quite as strongly, while the non-oxidative salts have no effect. The assumption that the mercuric effect is due to direct oxidation is therefore confirmed. The sequence of colors proves that the oxidation goes considerably beyond the stage of biliverdin, to the formation of more or less of the blue compound. As we are not prepared to take sides on the question whether there is only one blue bile derivative, we hesitate to use the term "bilicyanin," especially as in our series it occurred always mixed with lower or higher oxidized bile pigments. We prefer therefore to designate the whole series of oxidation products as "oxidized bilirubin," qualified by the adjective of the color that is actually observed.

*Effect of Acidity, of Temperature, and of Metallic Salts on Oxidation of Bile Pigments by Hydrogen Dioxide.*—Hydrogen dioxide in acid solution oxidizes bilirubin through the whole oxidative scale of yellow-green, dark green, blue, violet, pink, and colorless. (Obermayer and Popper, 1908, credit the description of this reaction to L. Grimbart, but we have not been able to consult his paper.) The oxidation is therefore profound, but it occurs quite gradually, its speed depending on conditions, thus constituting a convenient and striking test object for the influence of these conditions on the oxidative action of peroxide. This may be illustrated by the influence of acidity and of temperature.

To test the effect of acid, concentrated  $\text{HCl}$ , in quantities varying from 0.1 to 3 cc., was added to 2 cc. of the alkaline alcoholic bilirubin solution. One set of these mixtures was used as a control; these turned dark yellow-green at once with 0.1 cc. of  $\text{HCl}$ , and this tint did not change with further addition of  $\text{HCl}$  to 3 cc. and with standing for 3 hours.

To the other set of tubes, there was added 1 cc. of concentrated

hydrogen dioxide solution containing about 30 per cent of  $\text{H}_2\text{O}_2$ . The results are illustrated by Table I.

*The effect of the temperature* was tested by comparing the changes in the mixture of 0.25 cc. of  $\text{HCl}$ , 2 cc. of alkaline alcoholic bilirubin solution, and 1 cc. of dioxide solution at three temperatures. At  $25^\circ\text{C}$ ., the color did not change in 3 hours, beyond the immediate dark green. At  $50^\circ\text{C}$ ., the color changed slowly to light blue by the end of 2 hours and did not change further in 3 hours. At  $100^\circ\text{C}$ ., the mixture passed rapidly through the scale of colors becoming completely decolorized within 2 minutes.

*The effects of metallic salts* on the dioxide oxidation were tested by adding 1 cc. of hydrogen dioxide solution to the mixtures of

TABLE I.

*Effect of Acidity on the Speed of Oxidation of Bilirubin by Hydrogen Dioxide.*

The experiments were performed at room temperature.

Amount of concentrated $\text{HCl}$ added to 2 cc. of bilirubin and 1 cc. of peroxide solution.	Color immediately after mixing.	Time required for oxidation to complete decolorization.
cc.		
0	Dark green.	$\infty$ *
0.1	" "	$\infty$ *
0.25	" "	Between 2 to 3 hrs.
0.5	" "	" 1 " $1\frac{1}{2}$ "
1.00	Blue-green.	" $\frac{1}{2}$ " $\frac{1}{2}$ "
1.5	"	" 2 and 15 min.
2.0	Blue.	Less than 2 min.
3.0	Violet.	" " 2 "

\* The dark green color is scarcely changed by the end of 3 hours.

1 cc. of concentrated  $\text{HCl}$ , 1 millimol of the metallic salt, 2 cc. of the bilirubin solution. The oxidative decolorization of the bile pigments (*i.e.* to the color of the metallic salt) was completed as follows:

Cupric sulfate,	between	5 and 15 min.
Manganous sulfate,	5	" 15
Ferric chloride,	5	" 15
Cobaltous nitrate,	15	" 30
Nickelous "	15	" 30
Control (no metal),	30	" 60
Zinc sulfate,	60	" 90

Cadmium sulfate, more than 120 min.

Mercuric chloride, " " 120 "

The cadmium and mercuric mixtures were still of a light blue-green tint at the end of 2 hours.

The study brings out the interesting observation that the effect of the metallic salts on the  $H_2O_2$  oxidation of bile pigments has no relation whatever to their direct oxidative effects. Of the metallic salts that were directly oxidative, cupric sulfate and nickelous sulfate also accelerate peroxide oxidation, but mercuric chloride greatly retards the peroxide. Zinc sulfate and cadmium sulfate, which also retard the peroxide, do not directly oxidize the bile pigments. We are not prepared to interpret these observations.

*The effects of cyanide, quinine, and caffeine on the peroxide oxidation were also observed.* The same arrangement was adopted as for the metallic salts, adding 200 mg. respectively of potassium cyanide, quinine sulfate, or caffeine. The latter did not completely dissolve. Control experiments had shown that these do not alter the yellow-green color of acid bilirubin after standing 3 hours. With addition of the peroxide, the unpoisoned control was completely decolorized between  $\frac{1}{2}$  and  $\frac{3}{4}$  hour. Quinine and caffeine postponed the decolorization to between 1 and  $1\frac{1}{2}$  hours, and potassium cyanide to about  $1\frac{1}{2}$  and 2 hours.

*Mercuric Salts of Bile Pigments.*—Since bilirubin and biliverdin behave like feeble acids and form soluble salts with alkalis and insoluble salts with alkaline earths and metals (Küster, Städeler) it appeared probable that the mercuric chloride, besides oxidizing the bilirubin, would also form mercuric salts with the pigments. This was confirmed by preparing these salts directly from the bilirubin, and determining their properties and their mercury content. For this purpose, 0.5 gm. of "raw bilirubin" was dissolved in a mixture of 10 cc. of N/10 sodium hydroxide and 50 cc. of methyl alcohol. The solution was filtered; the excess of alkali was neutralized by adding N/10 acetic acid until a slight green shade appeared. The solution was again filtered and a solution of 2.0 gm. of mercuric chloride in about 50 cc. of methyl alcohol was added; the typical blue-green color appeared immediately and the addition of ether precipitated a dark green com-



pound. This precipitate was filtered off, washed successively with hot water, a small amount of alcohol, and ether, and dried in the desiccator.

The green compound is slightly soluble in alcohol and chloroform and practically insoluble in water, ether, and toluene. It is decomposed by dilute sodium hydroxide, and hydrogen sulfide precipitates mercury sulfide from the alcoholic solution.

The mercury content of this compound was kindly determined for us by Miss Nora E. Schreiber, using the method described by Booth, Schreiber, and Zwick. It gave the following results:

	Substance. mg.	Mercury. mg.	Mercury. per cent
1.	10.1	3.34	33.7
2.	6.6	2.10	31.9
	10.2	3.33	32.64
3.	10.0	2.86	28.6
	10.1	2.91	28.8

These values were found in three different preparations made in the same way. The difference of the analytical values is not greater than would be expected, in view of the fact that a slight excess of alkali would precipitate inorganic mercury, while a slight excess of acid in the preparation would precipitate a part of the bilirubin. The mercury content is not changed by prolonged washing with distilled water, so that it is evidently not held by mere adsorption. On the other hand the mercury content approximates the 26 per cent demanded by the theory for mercuric bilirubinate, analogous to Küster's calcium bilirubinate  $\text{CaC}_{32}\text{H}_{34}\text{O}_6\text{N}_4$ , which would correspond to 24.9 per cent for mercuric biliverdinate.

Attempts to isolate the mercury oxidized bilirubinate directly from bile have not been successful, because the presence of other substances interferes with the isolation.

#### CONCLUSIONS.

The deep blue-green color produced by adding mercuric chloride to bile (for instance, the Schmidt test for bilirubin in the feces) is not due to biliverdin but to a mixture of green and blue oxi-

dized bilirubin which exists as the mercury salt. The mercury content of this compound as prepared directly from bilirubin corresponds practically to that demanded by theory. It is a salt insoluble in ether, water, and toluene, slightly soluble in alcohol and chloroform. The mercury oxidized bilirubin is decomposed by alkali; its mercury is precipitated by hydrogen sulfide.

The color changes of the bile pigments form a convenient index of oxidative intensity: with air, the oxidation of the reddish bilirubin proceeds to yellow-green; with oxidizing metallic salts to dark green, and in the case of the mercuric chloride to blue-green; with hydrogen dioxide it progresses through the whole scale of dark green, blue-green, blue-violet, and pink to colorless. The speed of this change depends on conditions. It is greatly increased by acidity or by temperature. Metallic salts may influence the speed of the peroxide oxidation in either direction, irrespective of the direct oxidative action of the metallic salt. Mercuric chloride, a quite active direct oxidizing agent, retards the peroxide oxidation. This is also restrained by cyanide quinine, and caffeine.

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# CHOLESTEROL, LECITHIN, AND FATTY ACIDS IN THE BLOOD OF NEW BORN MICE WITH INHERITED ANEMIA AND THEIR NORMAL LITTER MATES.

BY S. B. DE ABERLE, W. M. HOSKINS, AND MEYER BODANSKY.  
(From the Departments of Anatomy and Biochemistry, Stanford University,  
California.)

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The inherited anemia discussed in this paper has been found to be caused by, or to be closely associated with, the gene  $W$  for dominant white (1). Mice of the genetic constitution  $Ww$  have a certain amount of white in their pelage, the recessives  $ww$ , in the absence of other factors for white, are uniformly colored, while the homozygous condition  $WW$  is manifested somatically by a severe anemia which terminates fatally a few days after birth. This trait acts as a simple Mendelian character. According to expectations one-fourth of all the offspring from  $Ww \times Ww$  matings are anemic and can be recognized as such by their pale appearance which contrasts strikingly with the livid color of their normal litter mates. They usually live from 3 to 5 days and die in an emaciated condition despite the fact that their stomachs can be seen to be gorged with milk.

Examination of several hundred anemic individuals has shown that they present a very constant picture. Their weight at birth averages 81.36 per cent of the normal. The anemic animal has only 15.04 per cent as many red corpuscles as the normal, and 25.24 per cent as much hemoglobin. The actual figures in terms of human standards are given in Table I.

The occasional normoblasts in the circulating blood show the same ratio in the two types of individuals. Showers of nucleated red cells, which are characteristic phases in the clinical picture of pernicious anemia, have never been observed. The number of reticulated red cells in the circulation of the anemic mice is

relatively higher than that in the controls, and the plastids showing reticulation are more heavily basophilic than in those of the normal. There is no leucocytosis in the anemic mice. The bone marrow is markedly aplastic with scattered blood cells lying in the swollen reticular network. When the fresh spleens of the two kinds of mice are compared side by side, the anemic spleens are approximately two-thirds the size of the normal, although there are slight individual variations. The anemic livers and thymuses are usually smaller than normal. The liver of the anemic individual, when examined in the living animal, is lighter in color than normal. No great difference between the visible fat content of the anemic and normal livers is revealed by frozen sections

TABLE I.  
*Some Characteristics of Normal and Anemic New Born Mice.*

	Weight.	Hemoglobin,*	Red cell count per 1 c.mm.
	<i>gm.</i>	<i>per cent</i>	
Anemic mice.	1.1353 (Average of 33 mice.)	22.5 (Average of 24 mice.)	676,730 (Average of 21 mice.)
Normal mice.	1.3953 (Average of 118 litter mates of the above.)	89.12 (Average of 27 mice.)	4,498,150 (Average of 18 mice.)

\* 100 per cent representing the normal for human blood. Color index for the anemic new born mice, 1.68.

stained with scharlach R. The normal new born mouse livers are invariably rich in lipid material which shows as round globules with a fat-soluble stain; the similar condition in the anemic liver, on the other hand, may or may not owe its origin to an abnormal fatty infiltration brought about by decreased oxidation in the parenchymatous tissue because of the lack of hemoglobin and oxygen in the blood.

The glycogen content in the livers from the two different individuals appears about the same when stained according to Best's technique.

Aside from the difference noted in the anemic hematopoietic tissue, a careful study of the other organs failed to reveal any characteristic which would distinguish them from the normal.

The placenta from the anemic and normal individuals at birth also appear similar on histological examination. Pieces of the anemic tissue transplanted into normal hosts have been able to survive, in some cases beyond the usual span of the anemic animal's life (2). All these facts point to the abnormality being due specifically to a deficiency in the blood-forming cells themselves.

Forty-two anemic mice and the same number of controls were used in the determination of cholesterol, lecithin, and fatty acids. The blood was collected in 10 cc. volumetric flasks containing a 3:1 alcohol-ether mixture until two samples, each containing 0.525 cc. of anemic blood, and two containing the same amount of normal blood were obtained. In each case a litter mate of the anemic mouse was used to insure the ages and environmental conditions being as nearly identical as possible. The time necessary to collect the material extended over a period of several weeks, owing to the small amount of blood that was available from the individual anemic mouse. Each anemic mouse was decapitated and the blood which flowed freely was measured with a calibrated micro pipette and then blown out under the solution in the flask. The same technique was used for the normal controls, taking from each the same amount of blood as had been procured from the anemic, to equalize any error that may have been due to the presence of small amounts of body fluids.

The methods employed for the determination of cholesterol and fatty acids were essentially micro adaptations of the methods of Bloor, Pelkan, and Allen (3). In the present work the amount of blood used for each analysis was much smaller than that called for in the original method, which necessitated reducing the quantity of each reagent. Before analyzing the blood of the mice, experience was gained and the accuracy of the modified methods ascertained by analyzing small quantities (0.3 to 0.5 cc.) of dog blood.

For the determination of lecithin, an aliquot portion of the alcohol-ether extract was placed in a small flask and evaporated on a water bath. 1 cc. of concentrated nitric acid and 6 drops of sulfuric acid were then added and the mixture heated over a micro burner in the manner recommended by Bloor (4). When the few remaining drops of high boiling liquid were perfectly

clear, the flasks were allowed to cool, the contents quantitatively transferred to a graduated tube, and the volume adjusted to 5 cc. The phosphorus present in this solution, derived from the phosphorus-containing lipoids of the alcohol-ether extract, was determined according to the method of Benedict and Theis (5). This method has been described for the determination of inorganic phosphorus in serum, but since the amount of non-lipoid phosphorus soluble in the alcohol-ether mixture is not great, the method may be successfully adapted for the determination of lipid phosphorus. Preliminary experiments showed that the intensity and persistence of the blue color developed in this procedure are dependent upon the amount of acid present and are improved by neutralizing the acid left from the digestion. In order to render the conditions uniform for the unknown and standard solutions, the following procedure was adhered to. After the solutions had been made up to 5 cc., as described earlier in this paragraph, 1.5 cc. of 1.0 M solution of potassium carbonate were added, followed by 2 cc. each of Benedict's hydroquinone-bisulfite reagent and Benedict's molybdic acid reagent. The solution was then placed in a tube and kept in a boiling water-bath for 10 minutes. After cooling, the color of the solution was compared colorimetrically with that obtained by treating similarly a standard phosphate solution of suitable concentration. The necessary correction was made for the phosphorus present as an impurity in the reagents.

The results for cholesterol, fatty acids, and phosphatides (expressed as lecithin), are given in Table II. It was found that the concentration of cholesterol in the blood of the new born anemic mice was much lower than in the blood of their normal litter mates. As is well known, cholesterol values comparable to these are frequently observed in pernicious anemia.

Usually, in man and dog, the total cholesterol of the blood is about equally distributed between the plasma and corpuscles. In anemia, the marked diminution of cholesterol in the plasma may be accompanied by normal values for the cholesterol in the corpuscles, or even by values higher than normal (compare Bloor and MacPherson (6), Pacini (7), Bodansky (8), and Bodansky and Dressler (9)). Unfortunately it is not known whether similar relations hold for the new born mice, as it was impossible

to secure, in this series of experiments, sufficient blood with which to study the distribution of cholesterol between the plasma and corpuscles. If the cholesterol in the blood of the new born mice were present primarily in the corpuscles, the low concentration in the blood could be explained on the basis of the low corpuscular content. On the other hand, these observations would have a different significance if the low cholesterol values were shown to be independent of the red cell count.

Slemons and Curtis (10), in a study of the cholesterol in the blood of mother and fetus (human), found, in nearly all cases, that all of the cholesterol in the fetal blood was present as free cholesterol, and not in combination as esters. Their values

TABLE II.

*Cholesterol, Lecithin, and Fatty Acids in Blood of Normal New Born Mice and in Blood of Those with Inherited Anemia.*

Specimen.	Cholesterol.	Lecithin.	Fatty acids.	Cholesterol Lecithin	Fatty acids Lecithin
	mg. in 100 cc.	mg. in 100 cc.	mg. in 100 cc.		
Normal. (Composite of 21 mice.).....	108.3	326	760	0.332	2.33
“ “ “ 21 “	114.7	316	696	0.363	2.20
Anemic. “ “ 21 “	77.1	329	855	0.234	2.60
“ “ “ 21 “	67.3	284	718	0.237	2.53

ranged between 110 and 225 mg. of cholesterol per 100 cc. of blood. Banu, Negresco, and Heresco (11) analyzed the serum of seven infants between the ages of 2 and 3 weeks and obtained an average value of 47 mg. of cholesterol per 100 cc. of serum. These observations, when considered together, point to an uneven distribution of cholesterol in the blood of the new born mammal.

The values for lecithin were approximately the same in the two kinds of mice. This was true, likewise, of the fatty acids, although one group of anemic mice gave a relatively high figure. The ratio, cholesterol : lecithin was lower than normal in the anemic mice; the ratio, fatty acids : lecithin was somewhat higher in anemia. Similar relations have been observed in clinical pernicious anemia (6).



## SUMMARY.

The form of inherited anemia discussed in this paper is manifested only in those mice having a specific combination of genes, *WW*. That the disturbed equilibrium of the organism is one not dependent on environmental conditions can be shown by the fact that the anemic and normal mice lie side by side in the uterus. The anemic may occupy any position with respect to the rest of the litter.

Taking the normal litter mate controls as a standard, the anemic mouse shows the following relations: the weight averages 81.36 per cent, the hemoglobin 25.24 per cent, the red corpuscles 15.04 per cent, the cholesterol 64.75 per cent, the lecithin 95.48 per cent, and the fatty acids 108.03 per cent of the normal.

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## PHENOL TESTS.\*

### III. THE INDOPHENOL TEST.

By H. D. GIBBS.

*(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)*

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#### INTRODUCTION.

Among the many methods of identifying phenolic compounds the test producing the beautiful, intense, blue solutions of the indophenol salts is probably the oldest. It dates back to the work of Robiquet (1835), J. Dumas (1838), R. Kane (1841), and other early workers, who were concerned with the orcein color substances, and who obtained the blue color with cresol, resorcinol, and other phenols without correct knowledge of the reactions involved, or of the composition of the color compounds. Lex (1870) and Weselsky (1871, 1872) obtained colors with nitrous acid and Kopp (1873) obtained the same color with fuming sulfuric acid, a test that is now known as the Liebermann test for phenols, for Liebermann (1874, 1875) showed that Kopp's fuming sulfuric acid contained nitrous acid.

Prior to 1874, complicated formulæ were proposed for the simple indophenols, and very little was known concerning their structure. The studies of von Baeyer and Caro (1874) resulted in the first approximation to the

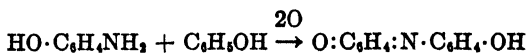
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true formula. The first commercially useful dyes of the class were prepared by Koechlin and Witt (1881, 1882) and patented by them in many countries.

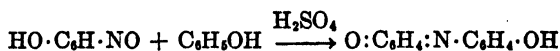
The indophenols and their closely related derivatives, the indamines, have been made by a large variety of methods<sup>1</sup> the most important of which may be classified by four types of condensations of two unlike molecules.

1. *Oxidation*.—By simultaneous oxidation of an amine and a phenol in the sense of the expression

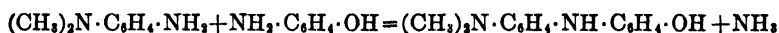


an indophenol is produced.

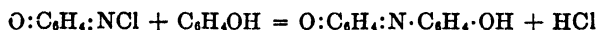
2. *Dehydration*.—The elimination of a molecule of water from a nitrosophenol and a phenol produces an indophenol.



3. *Deammonization*.—Certain amines and aminophenols may be condensed with the elimination of a molecule of ammonia forming a leuco derivative according to the reaction



4. *Deacidation*.—The coupling of 2 molecules with the elimination of an acid may produce an indophenol. The most important reactions of this type are those occurring between quinonechloroimides and phenols in the sense of the equation



Hirsch (1880) first described the condensation of quinonechloroimide and phenol and this method has been found to be the most satisfactory for the laboratory production of a great many derivatives of indophenol. The development of this type of reaction as a very delicate qualitative, and a very accurate quantitative, method for the estimation of phenol forms the substance of this paper. The procedure may be applied to many

<sup>1</sup> This subject will be more fully treated in a later paper on the synthesis of indophenols and indamines.

other phenols but only the quantitative data concerning phenol are presented.

It has been found that the dihalogen substituted quinonechloroimides, 2,6-dichloroquinonechloroimide and 2,6-dibromoquinonechloroimide give the most delicate tests of any of the quinonechloroimides tried and the indophenols formed from them are, in general, the most stable. In addition to these two, there have been investigated quinonechloroimide, *o*-cresolquinonechloroimide, and *m*-cresolquinonechloroimide.

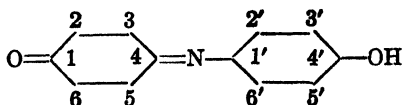
The test employing the 2,6-dibromoquinonechloroimide, has a delicacy of at least 1 part of phenol in 20,000,000. Further investigations may greatly extend its delicacy and usefulness. The indophenol formation has been followed quantitatively by means of the spectrophotometer and a number of absorption curves are charted.

The fact that the best results are obtained in buffered solutions should not mitigate against the use of this test for it is becoming more and more essential for a well equipped laboratory to be provided with a series of accurately standardized buffering solutions. Palitzsch's (1915, 1916) borax buffer solution, having a pH value of 9.24, may be employed. It is easily prepared by dissolving 19.108 gm. of sodium borate  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  in 1 liter of water.

The quinonechloroimides do not react with all phenols. It is generally believed that the primary requisite is that the position para to the hydroxyl must be unsubstituted (see Gibbs, 1926, 1927). This may be taken as a general rule although exceptions may be discovered when a larger number of phenols has been studied. Even with the para position free some substituted phenols have failed to react, due to the influence of the adjacent substituted groups.

A series of 2,6-dibromophenol indophenols has been investigated in this laboratory by Cohen, Gibbs, and Clark (1924) and a series of 2,6-dichlorophenol indophenols by Gibbs, Cohen, and Cannan (1925), and it has been found that these compounds form a series of easily made, stable indophenols. Since they were made from 2,6-dichloro- and 2,6-dibromoquinonechloroimide they were named, in the papers above referred to, the 2,6-dichloro-

and 2,6-dibromoindophenols, the numbering of the rings being as follows:



In a paper on the indophenols and indamines now in preparation the question of nomenclature will be fully discussed.

#### *Qualitative Test.*

Since the quinonechloroimides are only slightly soluble in water (see section on solubility) it is fortunate that only small concentrations are required for the test as applied to phenols. The quinonechloroimide reagent may be employed as an aqueous suspension or may be filtered to a clear solution. In the latter case a larger volume is required.

A mass of the solid 2,6-dibromoquinonechloroimide (or the dichloro derivative) about the size of a pea is shaken with 10 cc. of water in a test-tube to make a cloudy, yellow suspension. The larger particles sink rapidly and by drawing a portion from the middle of the suspension, into a pipette, the few drops of the reagent required for the test are readily delivered from the pipette into the solution to be tested. This procedure assures an excess of the imide which will increase the speed of the formation of the indophenol.

The quinonechloroimide decomposes slowly in alkaline buffers giving rise to discoloration of the solutions, which colors by no means should be mistaken for an indophenol test by one experienced with the reaction. The solutions to be tested should be very dilute, not stronger than 1 or 2 parts of the phenol in 1000 parts of solution, should be brought to an alkalinity ranging between pH 8 to 10, about pH 9.4 is preferable. 2 or 3 drops of the test solution, carrying some of the quinonechloroimide in suspension, are added to 10 to 50 cc. portions of the solution to be tested. In the presence of reacting phenols the blue color of the indophenol develops, in the more concentrated solutions intense blue almost instantly, and in very dilute solutions, 1 part in 20,000,000 or more; a pale blue may require an hour or

more for full development. Different indophenols require different pH for best formation but all are formed in the alkaline region. The best results are obtained by working in buffered solutions in color comparison tubes, and observing the color formation in a layer of solution 10 to 20 cm. in depth.

This test may be made approximately quantitative by comparison with known standards.

#### *Quantitative Method.*

The quantitative determination is best made by measurements of the color formation by means of the spectrophotometer. For phenol the readings are made at  $610\text{ m}\mu$ , the peak of the absorption band for this indophenol. When employing the Keuffel and Esser color analyzer 10 cm. observation tubes may be employed. By means of rough preliminary experiments, the solution to be tested should be brought to about a concentration of  $5 \times 10^{-6}\text{ M}$  with a buffering solution of about pH 9.4.

To 20 cc. of this solution in a test-tube, 2 or 3 drops of the 2,6-dibromoquinonechloroimide suspension, described in the qualitative procedure, are added, and the 10 cm. tube filled with this mixture.

The color formation may then be observed at time intervals measured in minutes until the maximum of absorption is shown. This requires from 10 to 20 minutes.

Where  $T$  is transmittancy,  $-\log T$  equals 1 for an indophenol concentration of  $5 \times 10^{-6}\text{ M}$  solution, observed through a 10 cm. layer. See "Absorption Spectra" described later in this paper.

#### *Quinonechloroimides.*

*a. Preparation.*—The two quinonechloroimides found to be most useful in this work are: (1) 2,6-dibromoquinonechloroimide and (2) 2,6-dichloroquinonechloroimide. They are readily prepared as follows:

A cold, hydrochloric acid solution of the corresponding amine, (1) 2,6-dibromo-*p*-aminophenol, (2) 2,6-dichloro-*p*-aminophenol, is slowly poured into a cold solution of sodium hypochlorite. Both solutions should contain much crushed ice and should be stirred constantly during the reaction. The addition of the

aminophenol solution should be stopped before a permanent blue or dark brown color appears and while free chlorine is still present.

The quinonechloroimides separate as canary-yellow precipitates which are collected on a Buchner filter, washed with a little water, and air-dried. When dry they are quite stable.

The solution of sodium hypochlorite is most easily prepared according to the method of Raschig (1907) as follows:

630 gm. of sodium hydroxide are dissolved in a little water, cooled, and poured onto sufficient crushed ice so that the total weight is 10 kilos. Chlorine gas is now run into the mixture in a rapid stream until the weight increases 730 gm. At this time much crushed ice should still be present. The solution of sodium hypochlorite is now ready for use. This quantity is sufficient for about 2.5 mols of the aminophenol.

*b. Solubility of 2,6-Dibromoquinonechloroimide.*—(With E. Elvove.) It has been found that the concentration of an aqueous solution of 2,6-dibromoquinonechloroimide can be determined most accurately by spectrophotometric measurement of the indophenol formed by the reaction with phenol in alkaline solutions. This reaction has been proved to be quantitative under certain conditions. A determination requires less than 20 minutes, the time depending upon the buffer solution employed. The solubility has been found to be 0.0002 M at 20°.

The saturated solution is prepared by shaking for about 10 minutes an excess of the compound, that has been powdered and sifted through a fine sieve, about 100 mesh, with water and filtering the suspension several times through a folded filter until the filtrate runs clear.

Care should be taken, however, to avoid too much powdering of the sample or grinding the powder together with the water in the mortar for too long a time, since it appears that under such conditions there is obtained what appears to be a supersaturated solution or a colloidal solution.

It has been found that 2,6-dibromoquinonechloroimide crystals undergo a slight decomposition on standing. This decomposition is more marked if the crystals are moist or in a moist atmosphere. Solutions change more rapidly, becoming deeply colored, a reaction which is accelerated by light. If kept in the dark the

solutions are serviceable for a number of hours. A nitrogenous decomposition product is formed in the crystals which is more soluble than the 2,6-dibromoquinonechloroimide. The preliminary attempts to check the spectrophotometric determinations of solubility by means of determinations of the nitrogen in the solutions and also the oxidizing power, was always found to give results very much too high, the former especially so. By repeatedly extracting the 2,6-dibromoquinonechloroimide with separate portions of water the greater part of this impurity was removed. The estimation of the amount of imide from the nitrogen determination and from the titration with thiosulfate of the liberated iodine then checked approximately in magnitude with the spectrophotometric determinations of indophenol formation. A sample of very carefully purified 2,6-dibromoquinonechloroimide that had been standing for several months in the laboratory in glass stoppered bottles, was found to contain an amount of this soluble impurity equivalent to about 0.06 per cent of the imide as calculated from the nitrogen determinations and compared with the imide as found by the spectrophotometer. The decomposition product does not interfere seriously with the accuracy of the indophenol determination although it is better to employ a second extraction in preparing the solutions for accurate work.

The three methods employed for determining the amount of 2,6-dibromoquinonechloroimide in solution are described as follows:

*Reduction Method.*—2,6-Dibromoquinonechloroimide liberates iodine from a solution of potassium iodide, and the titration of the iodine with standard thiosulfate solution gives a rough measure of the amount of the imide.

To 500 cc. of a saturated solution of the 2,6-dibromoquinonechloroimide there are added 100 cc. of a 5 N solution of hydrogen chloride, and then 5 cc. of a 20 per cent solution of potassium iodide. After thoroughly mixing, the liberated iodine is titrated with 0.1 N sodium thiosulfate.

1 mol of the imide should require 4 equivalents of hydrogen for complete reduction. This procedure has not been studied sufficiently to recommend it as an accurate method.

*Nitrogen Determination.*—It has been found most convenient



to determine the nitrogen in the solution after the thiosulfate titration of the iodine as described in the previous method. The Kjeldahl method was employed, always using 500 cc. portions of the 2,6-dibromoquinonechloroimide solution, and all determinations were made in duplicate.

*Spectrophotometric Method.*—This method of estimation of the 2,6-dibromoquinonechloroimide depends upon the spectrophotometric determination of the maximum blue color of the indophenol formed when a measured quantity of the imide solution reacts upon phenol in an alkaline solution.

The reaction proceeded very smoothly in a buffered solution of pH 9.0 to 9.5. Buffer of pH 9.4 was usually employed and the concentration of the phenol was at least 10 times that of the imide. The following procedure was found most satisfactory.

About 40 cc. of buffer solution pH 9.4 were put into a 50 cc. measuring flask, 5 cc. of a phenol solution of about  $4 \times 10^{-4}$  molality added, and then 1 cc. of the 2,6-dibromoquinonechloroimide solution was delivered into it from a standardized pipette, and then the flask was filled to the mark with the buffer solution. Thorough mixing was then assured by pouring the contents of the measuring flask into a 100 cc. Erlenmeyer flask from which spectroscopic tubes, 10 cm. in length, were filled. These tubes were put in place in the spectrophotometer and readings taken at intervals, at wave length  $610 m\mu$ , the peak of the absorption band for this indophenol. The maximum color usually develops in about 18 minutes. The reaction has a high temperature coefficient and may take longer if the temperature is below  $20^{\circ}$ .

In order to illustrate the points brought out in the previous discussion a series of determinations is described as follows and recorded in Table I.

40 gm. of an excellent sample of 2,6-dibromoquinonechloroimide were extracted consecutively with 3 portions of water of 1200 cc. each, the temperature of which was approximately  $20^{\circ}$ , and filtered to clear solutions.

Duplicate 500 cc. samples of each extraction were taken for the iodine titration and the nitrogen determination and 1 cc. portions were taken for the spectrophotometric measurements.

The spectrophotometric method is by far the simplest, quickest, and most accurate. The other methods merely show the magni-

tude of the agreement and the interference of other soluble compounds with the titration methods that do not interfere with the indophenol formation.

The spectrophotometric determinations agreed perfectly in duplicate. The three extractions differed slightly in the length of time of extraction and in the temperature of the extraction. The fact that the concentration is greater in the second and third extraction is of no significance but due entirely to the difference

TABLE I.  
*Determination of 2,6-Dibromoquinonechloroimide in Solutions.*

Extraction No.	Temperature.	Iodine titration.		Nitrogen by Kjeldahl.		Spectrophotometric indophenol estimation.			
		0.1 N thiosulfate.	2,6-Dibromoquinonechloroimide equivalent in molar solubility.	0.1 N acid.	2,6-Dibromoquinonechloroimide equivalent in molar solubility.	Buffer.	T transmittancy.	- log T.	2,6-Dibromoquinonechloroimide equivalent in molar solubility.
	°C.	cc.		cc.		pH			
I	19	13.2	0.00066	11.17	0.00223	9	0.21	0.678	0.0001695
						9.4	0.21	0.678	0.0001695
II	19	8.55	0.00048	2.82	0.00056	9	0.20	0.699	0.0001748
						9.4	0.20	0.699	0.0001748
III	22	6.05	0.00030	1.49	0.00030	9.4	0.145	0.839	0.0002097

in manipulation. The temperature of the first extraction was 19° and the third 22°.

The unit value for -log T has been found to be equivalent to a concentration of indophenol of  $5 \times 10^{-6}$  M as demonstrated in another part of this paper.

#### *Absorption Spectra.*

Absorption spectra of the sodium salts of 2,6-dichloro- and the 2,6-dibromoindophenols are plotted in Fig. 1, and for purposes of comparison the curve for methylene blue is introduced in the same figure.

These products have been described in the prior work of this laboratory and the analyses showed

	Purity. per cent
2,6-Dichloroindophenol.....	72.9
2,6-Dibromoindophenol.....	85.5
Methylene blue, Sample F.....	76.6

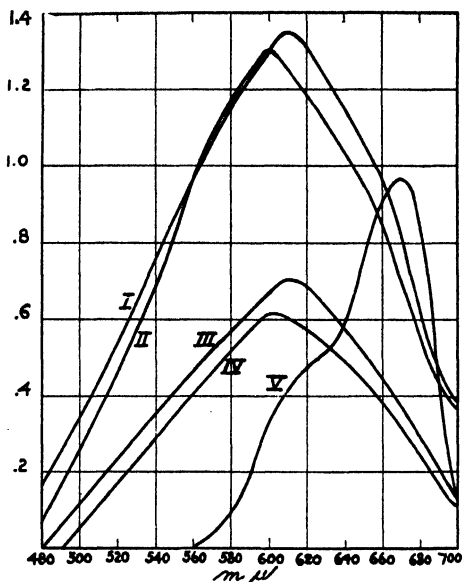


FIG. 1. Spectrophotometric absorption curves of the sodium salts of: I, 2,6-dibromoindophenol 0.00001 M; II, 2,6-dichloroindophenol 0.00001 M; III, 2,6-dibromoindophenol 0.000005 M; IV, 2,6-dichloroindophenol 0.000005 M; V, methylene blue chloride 0.000002 M. The ordinates are measured as  $-\log T$ .

The only impurities in the indophenols are sodium chloride and water and the methylene blue is approximately pure except for water. The latter was most highly purified as described by Clark, Cohen, and Gibbs (1925).

The absorption curves are plotted from data obtained from the study of unbuffered solutions. These were made by dissolving a molar weight of the compound in mg. without correcting for the purity, in 1000 cc. of water and further diluting this to

the desired concentration as stated in the chart. The absorption of the solutions was measured in 10 cm. tubes.

It is to be noted that the absorption of equal molar solutions of the dichloro and the dibromo compounds is almost equal while the peaks of the curves occur at different wave lengths; namely, 600  $m\mu$  for the chloro and 610  $m\mu$  for the bromo derivatives. Equimolecular solutions are quite different in quantity of material present being in the ratio of  $\frac{\text{chloro}}{\text{bromo}} = \frac{289}{379} = \frac{1}{1.31}$ .

While the absorption of methylene blue is in quite a different region of the spectrum it is noticeable that the coloring power, as measured by the peak of the absorption band, is much greater, the E values being in about the proportion of 0.7 for the indophenol to 2.5 for methylene blue.

It is readily seen from the absorption curves that methylene blue is a green-blue while the indophenols are a much purer blue.

Too much reliance is not to be placed upon the statements regarding the relative persistence of the peaks of the indophenols when measured in unbuffered solutions since these compounds act as acid-base indicators and are sensitive to the carbon dioxide of the atmosphere.

An accurate determination of the peak of the absorption band of 2,6-dibromindophenol at 610  $m\mu$  places the value of  $-\log T$  at 1 for solutions  $5 \times 10^{-6}$  M in buffer pH 9 when measured in 10 cm. tubes. The deviations from this value, due to pH in the range from pH 8.5 to 10, are very slight and do not affect the value materially. The standard solution,  $5 \times 10^{-5}$  M, was prepared by dissolving the proper quantity of the indophenol, of 85.5 per cent purity previously mentioned, in water. 5 cc. portions of this solution were put into a 50 cc. flask which was filled to the mark with buffer pH 9, thus making a solution  $5 \times 10^{-6}$  M from which the 10 cm. tubes were filled for the spectrophotometric observations.

#### *Effect of pH upon the Rate of Indophenol Formation.*

Very early in the investigation it was noted that the pH of the solution greatly affected the rate of the formation of the blue color. The reaction velocity increases with the increase in

alkalinity. A comparison of the rates at pH 8.5, 9, 9.5, and 10 was made by treating a  $5 \times 10^{-6}$  M phenol solution in these buffers with an excess of 2,6-dibromoquinonechloroimide, as described under the analytical procedure, and observing the indophenol formation as measured in the spectrophotometer at  $610 \text{ m}\mu$  at time intervals.

2 gm. of pure crystals of phenol were put into water with 50 cc. of 0.2 N sodium hydroxide (about one-half the quantity to form sodium phenolate) and the solution was made to 2 liters. It was clear and colorless. 10 cc. of this solution were diluted to 1 liter, thus making a solution which contained 1 part of phenol in 100,000, or  $0.0001064 \text{ M}$ . This was used for a stock solution and diluted to varying degrees in buffers for tests. When 1 cc. of this solution was put into 20 cc. of buffer there resulted a solution of the concentration of  $5 \times 10^{-6} \text{ M}$  or 1 part of phenol in 2,100,000. This concentration was employed in determining spectrophotometrically the effect of pH upon the speed of the reaction.

To 21 cc. of the buffered solution in a test-tube 2 or 3 drops of a 2,6-dibromoquinonechloroimide suspension were added. As soon as added the contents of the test-tube were mixed and the 10 cm. tube, in which the absorption is measured, was filled and placed in the spectroscope and readings taken at once.

The first readings were low, due to the slight turbidity of the solution, but soon the solution cleared and read 100 per cent transmittancy. Then the color began to develop and the absorption, due to the indophenol formation, was read at the wavelength  $610 \text{ m}\mu$ .

The time required for the clearing of the solution and the beginning of the indophenol blue formation, as observed by the spectrophotometer, was quite different in the different buffers, being 16 minutes at pH 8.5, 7 minutes at pH 9, 3 minutes at pH 9.5, and 2 minutes at pH 10. The solubility of the quinonechloroimide and also the rate of decomposition increases with the pH; that is, with the increasing alkalinity. Discoloration of the solution slowly takes place at the higher alkalinities.

In several instances, near the end of the reaction, the full curves were plotted and they coincide very nearly with the curves for the purified compounds plotted in Fig. 1.

TABLE II.

*Spectrophotometric Data of Formation of Indophenol from 2,6-Dibromoquinonechloroimide and Phenol in Solutions of Concentration of 1 Part in 2,100,000 of 0.000005 M at Various Degrees of Alkalinity.*

These results are plotted in Fig. 2.

T = transmittancy.

pH 8.5			pH 9			pH 9.5			pH 10		
Time -16 min.	T	- log T	Time -7 min.	T	- log T	Time -3 min.	T	- log T	Time -2 min.	T	- log T
0	1.00	0.0000	0	0.99	0.0044	0	0.99	0.0044	0	0.99	0.0044
10	0.93	0.0315	3	0.91	0.0410	1	0.92	0.0362	2	0.85	0.0706
22	0.85	0.0706	6	0.86	0.0655	3	0.85	0.0706	4	0.70	0.1549
65	0.60	0.2217	11	0.72	0.1427	5	0.79	0.1024	7	0.54	0.2676
102	0.43	0.3665	16	0.60	0.2218	7	0.67	0.1739	10	0.42	0.3768
135	0.32	0.4948	21	0.50	0.3010	9	0.63	0.2007	12	0.32	0.4949
176	0.27	0.5686	26	0.44	0.3565	11	0.60	0.2217	14	0.29	0.5376
220	0.22	0.6576	31	0.38	0.4202	13	0.55	0.2596	17	0.24	0.6198
252	0.185	0.7328	34	0.35	0.4559	15	0.47	0.3279	22	0.15	0.8239
285	0.16	0.7959	49	0.18	0.7447	17	0.40	0.3979	27	0.13	0.8861
1685	0.12	0.9208	58	0.16	0.7939	19	0.36	0.4437	32	0.115	0.9393
After this time the rate of fading is so great that the maximum color is not developed.			71	0.11	0.9586	21	0.31	0.5086	40	0.09	1.0458
			101	0.10	1.0000	23	0.28	0.5528	47	0.088	1.0555
			121	0.09	1.0458	25	0.25	0.6021			
						27	0.23	0.6383			
						29	0.21	0.6778			
						31	0.19	0.7212			
						36	0.16	0.7959			
						41	0.13	0.8861			
						46	0.12	0.9208			
						51	0.11	0.9586			
						61	0.105	0.9788			
						86	0.09	1.0458			

The data are tabulated in Table II and plotted in Fig. 2. The zero point is taken at the beginning of the indophenol formation where the transmittancy = 1. The inhibition period mentioned above is omitted from the time.

It is to be noted that the complete indophenol formation at pH 9, 9.5, and 10, from the total amount of phenol present, is shown by the curves.

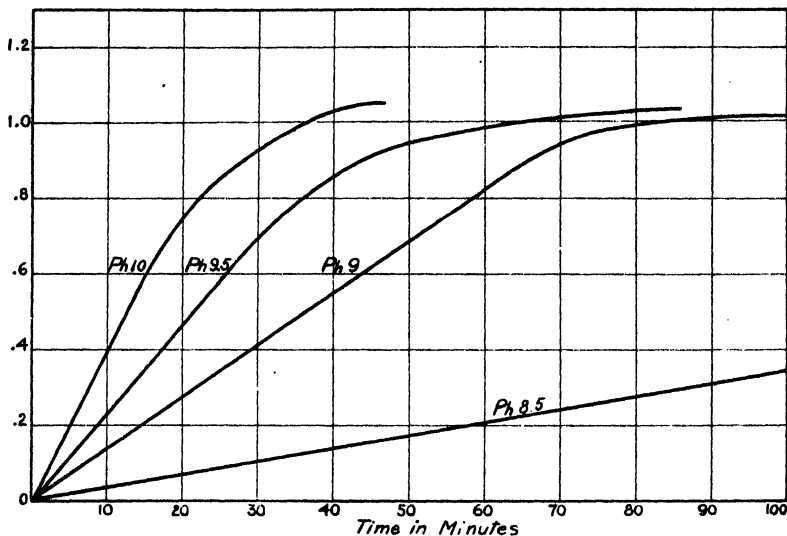


FIG. 2. Graphic representation of the data recorded in Table II, showing the formation of 2,6-dibromoindophenol at various degrees of alkalinity as measured by the spectrophotometer readings at  $610m\mu$ , the peak of the absorption band of this indophenol. The ordinates are measured as  $-\log T$ .

A quantitative study of the reaction velocity together with a study of the underlying causes in the change in speed due to pH and a mathematical treatment of the same forms the subject of the next paper of this series.

#### SUMMARY.

A test for phenols, depending upon the quinonechloroimide reaction producing indophenols, is described. Qualitative data for phenols are given and it is shown that the test is made quanti-

tatively accurate by following the color formation by means of the spectrophotometer. The test is delicate, both qualitatively and quantitatively, to 1 part of phenol in 20 million by ordinary manipulative skill. The effect of the pH of the solution on the velocity of indophenol formation is shown.

Almost all phenols having the position para to the hydroxyl group unsubstituted, and also some other derivatives such as the amines, will form indophenols (or indamines) and, therefore, the method will be useful for differentiating between phenols only on the basis of the absorption spectra. This phase of the test is not treated in this paper.

A method for the preparation of the reagent is given and the solubility of the best reagent, 2,6-dibromoquinonechloroimide, has been determined with considerable accuracy.

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## THE BUFFERING OF THE TISSUES AS INDICATED BY THE CO<sub>2</sub> CAPACITY OF THE BODY.

BY R. J. BROCKLEHURST AND YANDELL HENDERSON.

(From the Laboratory of Applied Physiology, Sheffield Scientific School,  
Yale University, New Haven.)

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It is common in the current literature to read of "the buffering of the blood and tissues." The words imply that the considerable information which has been won in recent years regarding the blood can be applied directly to the tissues. In fact, however, comparatively little is known regarding the effectiveness with which the tissues are buffered.

The most significant expression of the buffering of the blood is afforded by the slope of the CO<sub>2</sub> dissociation curve. In this paper we shall refer only to the CO<sub>2</sub> dissociation curve for uniformly oxygenated blood, and to the slope of this curve within the physiologically significant region between 40 and 50 mm. partial pressures of CO<sub>2</sub>. On examining the much used curve for Haldane's blood (1) and comparing the curves obtained by other workers (2), we find that the variations are slight: for all normal bloods the slope between 40 and 50 mm. is nearly the same. The figure for this slope is 3.42 volumes per cent of CO<sub>2</sub> taken up or given off for a change in the partial pressure of CO<sub>2</sub> of 1 per cent of an atmosphere. At higher pressures the slope is slightly less, at lower pressures it is more; but as our measurements both at higher and lower pressures start from the normal value we have used the figure 3.42 as the standard with which to compare our data.

It occurred to us to determine the corresponding figure for the entire body of a living man. For this purpose we have used two methods which may be termed respectively: (1) the accumulative method, and (2) the eliminative method.

*Accumulative Method.*

The amount of CO<sub>2</sub> (calculated to standard temperature and pressure) that the body is producing per minute is determined in the ordinary way. Then the subject, after exhaling as deeply as possible, starts breathing at natural rate and depth from and into a rubber bag holding 7 liters of a mixture of oxygen and 5

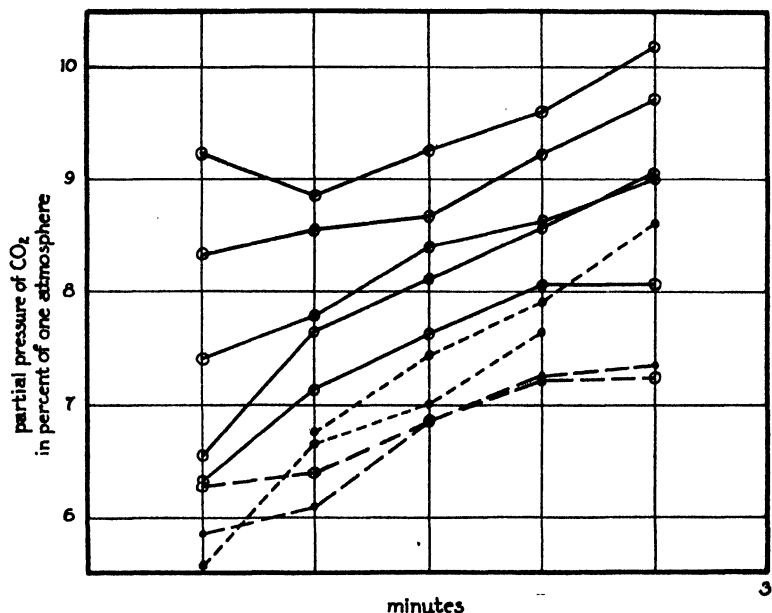


FIG. 1. Showing the analyses of the samples from a number of experiments. Differences in the height of the several curves are due to different initial concentrations of CO<sub>2</sub> in the bag; in at least one instance it can be seen that CO<sub>2</sub> passed first from the bag into the blood. The solid, dash, and dotted lines indicate observations on R. J. B., H. W. H., and Y. H. respectively.

per cent or more of CO<sub>2</sub>. The oxygen supply is always ample throughout the experiment, as indicated both by the color of the lips and by analysis; the hemoglobin in the blood is therefore fully oxygenated, and only the relations of CO<sub>2</sub> in uniformly oxygenated blood are involved. At intervals of 30 seconds samples are drawn from the bag close to the mouthpiece and as nearly as possible at the end of an expiration, and analyzed.

The rate at which the pressure of  $\text{CO}_2$  rises in the rebreathed oxygen is thus determined. The data of nine experiments on three men are shown in Fig. 1. For the  $2\frac{1}{2}$  minutes that the experiment continues and for pressures between 6 per cent and 10 per cent of an atmosphere, the rise is fairly uniform for each individual, and the average slope of each of these curves is the basis upon which the figures in Column 3 of Table I are calculated. The rate at which  $\text{CO}_2$  accumulates in the system, consisting of man and bag, is evidently identical with the  $\text{CO}_2$  production of the body; and the accumulation in the body is less than that in the whole system by the quantity accumulating in the bag. When the figure for the amount of  $\text{CO}_2$  accumulating in the body in 1 minute is divided by the body weight, we obtain the increase of  $\text{CO}_2$  content in the body in terms of volumes per cent per minute. From the analyses of the contents of the bag the corresponding rise of pressure of  $\text{CO}_2$  per minute is obtained. By combining the two quantities—increase of content and rise of pressure in equal time—essentially the same information is obtained regarding the entire body that is obtained regarding the blood from a series of equilibrations and analyses in the usual procedure for determining the  $\text{CO}_2$  dissociation curve. This is the first step toward estimating the capacity of the tissues other than blood.

It is not claimed that each of the analyses of the carbon dioxide in the bag represents the pressure of this gas in the body at the instant that the sample is taken. But it is assumed that the rate at which the pressure of carbon dioxide rises in the bag is the same as the rate of rise in the body; that is, the lag is constant.

Having obtained these data for the rise of the content of  $\text{CO}_2$  of the whole body in volumes per cent per minute, and for the rise of pressure in per cent of an atmosphere per minute, we then calculate the rise of content in volumes per cent per 1 per cent of an atmosphere. This figure includes two quantities: (1) the  $\text{CO}_2$  that has accumulated in the blood per 1 per cent of an atmosphere rise of pressure, and (2) the  $\text{CO}_2$  which accumulates in the tissues under this increase of pressure. It is difficult to estimate the allowance that should be made for the first of these quantities; but we have assumed (after consulting Erlanger's review (3)) that the mass of blood in active circulation is about 5 per cent of the body weight. Accordingly, as  $3.42 \times 0.05 = 0.17$ , this

## Protocol 1.

Subject, weight 62 kilos.

CO<sub>2</sub> production at rest..... 214 cc. per min.

Bag (7 liters), containing O<sub>2</sub> and CO<sub>2</sub>, and residual air (1.5 liters) = 8.5 liters.

Commenced rebreathing into bag; gas samples withdrawn every 30 seconds.

Analyses for CO<sub>2</sub> in per cent: (1) 7.42, (2) 7.79, (3) 8.4, (4) 8.62, (5) 9.0

Average rise of CO<sub>2</sub> in bag = 0.79 per cent per min.

Equivalent to an accumulation in the bag of CO<sub>2</sub> production per minute less CO<sub>2</sub> accumulation in bag per minute = 214 - 61 = 153 cc. per min.

(this figure representing the accumulation of CO<sub>2</sub> in the body)

Expressing this in terms of volumes per cent,

we have  $153 \times \frac{100}{62,000}$  = 0.247 vols. per cent per min.

Or, in terms of volumes per cent per 1 per cent

atmosphere  $\frac{0.247}{0.79}$  = 0.313 vols. per cent per 1 per cent atmosphere.

CO<sub>2</sub> capacity of blood, from the dissociation curve between 40 and 50 mm. CO<sub>2</sub>

= 3.42 vols. per cent per 1 per cent atmosphere.

Assuming that the mass of the circulating blood is 5 per cent of the total body weight, the CO<sub>2</sub> capacity of the remaining body tissues is

$\frac{0.313 - (3.42 \times 0.05)}{0.95}$  = 0.149 vols. per cent per 1 per cent atmosphere.

If the blood mass is 7.5 per cent of the body weight, the CO<sub>2</sub> capacity would be

$\frac{0.313 - (3.42 \times 0.075)}{0.925}$  = 0.0575 vols. per cent per 1 per cent atmosphere.

fraction of a volume per cent is subtracted from the figure for the accumulation in the whole body. Then the remainder is divided by 0.95 to obtain the average rise of CO<sub>2</sub> in volumes per cent per 1 per cent of an atmosphere in all the tissues of the body

other than blood. In Protocol 1 is given an example of such an experiment. In Table I are shown the results of several experiments of this sort on each of three men.

The results of these experiments are shown graphically in Fig. 2, where the CO<sub>2</sub> capacity of blood and of separated plasma are also represented. They indicate that the capacity of the tissues is far below that of the blood. Even if we assume that the skin,

TABLE I.

Subject.	CO <sub>2</sub> production at rest.	Average rise of CO <sub>2</sub> .	Output of CO <sub>2</sub> in bag.	CO <sub>2</sub> accumulating in body.			CO <sub>2</sub> capacity of body (excluding blood.)*
				cc. per min.	vols. per cent per min.	vols. per cent per 0.01 atmosphere	
R. J. B. 62 kilos.	262	0.80	61.5	200.5	0.323	0.404	0.246
	205			143.5	0.232	0.290	0.125
	250	0.81	62.7	187.3	0.302	0.373	0.213
	214	0.79	61.0	153.0	0.247	0.313	0.149
	238	0.78	60.2	177.8	0.286	0.366	0.206
		0.87	66.8	171.2	0.276	0.318	0.155
	Average.						0.182
H. W. H. 90 kilos.	368	1.02	83.6	284.4	0.316	0.310	0.146
	328	1.19	95.6	232.4	0.258	0.217	0.049
	Average.						0.098
Y. H. 82 kilos.	229	0.84	69.3	159.7	0.195	0.238	0.071
	267			197.7	0.241	0.294	0.129
	241	0.67	54.1	186.9	0.228	0.340	0.178
	Average.						0.126

\* Assuming blood mass to be 5 per cent of body weight.

bones, and other inactive tissues make up half the weight of the body, the order of magnitude of the capacity for CO<sub>2</sub> of the muscles and viscera appears still to be below even that of separated blood plasma. The capacity to hold carbon dioxide in considerable amounts under a slight rise of partial pressure is a striking property of whole blood. It is relatively low in plasma from which the corpuscles have been separated. It is here shown to be still lower in the tissues.

Measurements by the method here described are not very exact, owing to the varying vascularity of the tissues, uncertainty as to the circulating blood volume, errors in sampling, etc. Nevertheless they are sufficient to afford a definite indication of the order of magnitude of the buffering of the tissues in comparison with blood and with separated plasma. The approximate relation is that more than one-half of the entire CO<sub>2</sub> capacity of the body is afforded by the blood, assuming that the blood forms only 5 per cent of the body weight. If a larger blood mass is assumed, say 7.5 per cent of the body weight, the capacity of the tissues must be estimated at a correspondingly lower figure; for a 7.5 per cent blood volume, two-thirds of the CO<sub>2</sub> capacity would be in the blood and only one-third in all the other tissues of the body.

#### *Eliminative Method.*

For this method the CO<sub>2</sub> production and the CO<sub>2</sub> pressure in the alveolar air at rest were first determined. Then the excess CO<sub>2</sub> production involved in the work of forced breathing was estimated by means of a Douglas bag in which accumulated the expired air during 1 minute of forced breathing and 2 or 3 subsequent minutes of spontaneous breathing. After these preliminary determinations the actual experiment consisted in performing forced breathing for 1 minute, while the expired air was collected in a Douglas bag for subsequent metering and analysis. The excess elimination of CO<sub>2</sub> over and above the amount which the body was producing was then determined. At the end of the forced breathing the alveolar CO<sub>2</sub> pressure was determined. From the data of CO<sub>2</sub> production and elimination and from the alveolar pressures at the beginning and end of the period of forced breathing, we have calculated the amount of CO<sub>2</sub> removed from the body per 1 per cent of an atmosphere fall of pressure.

An example of one of these experiments is given in Protocol 2. The data of several such experiments and the average results for each subject are given in Table II.

It will be seen that the results obtained by the eliminative method are in close agreement with those of the accumulative method, and that they also indicate therefore a remarkably small CO<sub>2</sub> capacity in the tissues.

# Protocol 2.

Subject, weight 62 kilos.	
CO <sub>2</sub> production at rest.....	203 cc. per min.
Resting alveolar air contained.....	4.84 per cent CO <sub>2</sub> .
Alveolar air after 1 minute's forced breathing contained.....	2.54 per cent CO <sub>2</sub> .
Therefore fall of CO <sub>2</sub> in lungs	= 2.30 " " per min.
Volume of air expired during 1 minute's forced breathing	= 57.65 liters
Containing 1.84 per cent of CO <sub>2</sub> , and $57.65 \times$ 0.0184	= 951 cc. per min.
Forced breathing for 1 minute followed by spon- taneous breathing for 3 minutes resulted in 60.8 liters of expired air, containing 2.11 per cent CO <sub>2</sub> , and $60.8 \times 0.0211$	= 1147 cc. per 4 min.
Resting CO <sub>2</sub> production of body = 203 cc. per min. or 812 cc. per 4 min.	
I.e. $1147 - 812 = 335$ cc. CO <sub>2</sub> are due solely to the exercise of 1 minute's forced breathing.	
Total volume of CO <sub>2</sub> eliminated from the body per minute during 1 minute of forced breathing = $951 - 335$	= 616 cc.
Volume of CO <sub>2</sub> eliminated from the body, in ex- cess of resting CO <sub>2</sub> production = $616 - 203$	= 413 cc. per min.
In terms of volumes per cent per minute this is $413 \times \frac{100}{62,000}$	= 0.667 vols. per cent.
In terms of volumes per cent per 1 per cent at- mosphere $\frac{0.667}{2.3}$	= 0.29 vols. per cent.
CO <sub>2</sub> capacity of blood (as before)	= 3.42 vols. per 1 per cent atmosphere.
If blood mass is 5 per cent of total body weight, CO <sub>2</sub> capacity of remaining tissues is $\frac{0.29 - (3.42 \times 0.05)}{0.95}$	= 0.125 vols. per cent per 1 per cent at- mosphere.
If blood mass is 7.5 per cent of total body weight, CO <sub>2</sub> capacity of remaining tissues is $\frac{0.29 - (3.42 \times 0.075)}{0.925}$	= 0.036 vols. per cent per 1 per cent at- mosphere.



## DISCUSSION OF RESULTS.

The results by both methods are shown graphically in Fig. 2. The CO<sub>2</sub> capacity of the tissues as determined by the accumulative method on three men is represented by the three lower solid lines. The capacity as determined on two of these men by the eliminative method is indicated by the two lower broken lines. The significance of these results appears when they are contrasted with the

TABLE II.

Subject.	CO <sub>2</sub> production at rest.			Fall of CO <sub>2</sub> .	CO <sub>2</sub> output during 1 min. forced breathing.		CO <sub>2</sub> eliminated from body.		CO <sub>2</sub> eliminated from body in excess of resting production.			CO <sub>2</sub> capacity of body (excluding blood).*
	cc. per min.	per cent	per cent		cc.	cc.	cc. per min.	cc. per min.	vols. per cent per min.	vols. per cent per 0.01 atmosphere	vols. per cent per 0.01 atmosphere	
R. J. B.	247	4.94	2.59	2.35	1230	450	780	533	0.860	0.366		0.206
62	216	5.23	2.85	2.38	971	360	611	395	0.637	0.268		0.102
kilos.	203	4.84	2.54	2.30	951	335	616	413	0.667	0.290		0.125
										Average.		0.144
Y. H.	254	4.65	2.29	2.36	590	245	345	91	0.111	0.470		0.315
82	194	4.45	2.04	2.41	504	237	267	73	0.089	0.370		0.209
kilos.										Average.		0.262

\* Assuming blood mass to be 5 per cent of body weight.

CO<sub>2</sub> capacity of the blood, separated plasma, water at 37°C., and plasma merely in its dissolved CO<sub>2</sub> without the combined CO<sub>2</sub>: all indicated by lines in the figure. In the calculations which lead to the values here assigned to the tissues, the CO<sub>2</sub> capacity of the blood is subtracted from that of the body as a whole. For the blood we have assumed a mass of only 5 per cent of the body weight. If a higher figure were used for the blood, the values

calculated for the  $\text{CO}_2$  capacity of the rest of the body tissues would be considerably lower even than they are here shown.

Considering all the analytical factors and the possible errors involved in determining such physiological functions as those here involved, we do not feel that the values for the  $\text{CO}_2$  capacity of the body apart from the blood are better than rough approximations. They are, however, probably of about the right order of

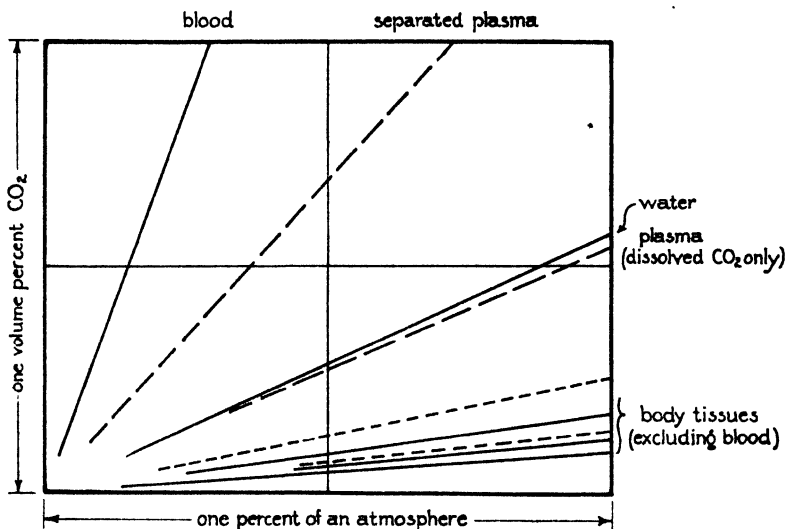


FIG. 2. Showing the relative  $\text{CO}_2$  capacity of blood, separated plasma, water at  $37^\circ\text{C}$ ., and plasma as a solvent, apart from combined  $\text{CO}_2$ . In contrast to these values are shown in the five lower lines the estimated capacities of the tissues of the body apart from the blood. Solid lines are from data obtained on three men by the accumulative method; broken lines from data obtained on two men by the eliminative method.

magnitude. The inclusion of bones and skin certainly decreases the general average for the body by a considerable amount. But even when every allowance is made, it appears probable that the  $\text{CO}_2$  capacity of the muscles and viscera is far below that even of separated plasma. It seems fair to conclude that at least half of the entire  $\text{CO}_2$  capacity of the body is afforded by the reaction of the hemoglobin of the blood in supplying base for the formation of bicarbonate under slight changes of pressure of  $\text{CO}_2$ .

Another inference is that the body of a normal human adult contains not more than 4 or 5 liters of CO<sub>2</sub>: a much smaller volume than would be the case if the capacity of the tissues were the same, kilo for kilo, as that of the blood. Presumably the relative contents of CO<sub>2</sub> in the blood and in the rest of the body are proportional to their capacity to take up and give off CO<sub>2</sub> under slight changes of partial pressure. Thus at least half the CO<sub>2</sub> content of the body is in the blood: the total content of CO<sub>2</sub> is roughly the same as the blood volume.

#### CONCLUSIONS.

The two methods here employed determine (1) the rate of rise of pressure of CO<sub>2</sub> in the body when a certain amount of CO<sub>2</sub> is accumulated, and (2) the rate of fall of pressure when the content of CO<sub>2</sub> is decreased. Thus they afford an indication of the relation of pressure and content of CO<sub>2</sub> in the whole body, essentially like that afforded by the slope of the CO<sub>2</sub> dissociation curve for blood. The CO<sub>2</sub> capacity of the body in volumes per cent per 1 per cent of an atmosphere partial pressure has thus been estimated on three men. This capacity is an index of the effectiveness of buffering.

The results indicate so low a capacity in the tissues that more than half of the total capacity for the entire body is due to the hemoglobin of the blood. When allowance is made for such tissues as bone, skin, etc., it still appears that the capacity of the muscles and viscera is probably considerably less than that of separated blood plasma.

*Addendum.*—After this paper had been sent in for publication we found that a few weeks previously a somewhat similar investigation had been published by Shaw (4). His observations were made on cats under artificial respiration. His conclusion is that the tissues afford 88 per cent of the CO<sub>2</sub> capacity of the body. This is a very different estimate from ours. No simple explanation for the disagreement occurs to us at this time, unless perhaps it may be found in the fact that our experiments lasted each for only 2 or 3 minutes, while those of Shaw occupied an hour or more, and small analytical errors might thus be magnified in his calculations.

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## STUDIES IN SERUM ELECTROLYTES.

### II. THE ELECTROLYTE COMPOSITION AND THE pH OF SERUM OF A POIKILOTHERMOUS ANIMAL AT DIFFERENT TEMPERATURES.

BY J. HAROLD AUSTIN, F. WILLIAM SUNDERMAN,\*  
AND J. G. CAMACK.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

(Received for publication, January 24, 1927.)

#### INTRODUCTION.

In discussing the effect of temperature variations on the blood, Austin and Cullen (1) called attention to the necessity of considering separately  $\alpha_{H^+}$ ,  $\alpha_{OH^-}$ , and  $\frac{\alpha_{H^+}}{\alpha_{OH^-}}$  and further of determining experimentally which of these is the important factor in the acid-base equilibrium under conditions of changing temperature.

In mammals the change in body temperature which can be produced without injury is small. Poikilothermous animals, however, in which changes of 15–20°C. are easily borne would be suitable for an investigation of this question.

With this object in view a survey of a number of poikilothermous species was made and the alligator finally chosen as most suitable for our purpose. For assistance and animals we are greatly indebted to Dr. Herbert Fox, to the Philadelphia Zoological Society, and to Mr. C. Emerson Brown, Director of their Garden.

#### *Methods.*

The studies were made on four alligators varying in weight from 4 to 6 kilos. The animals except for their heads and a small area of their backs were kept immersed in water for 3 days preceding each bleeding and the water during this time was kept

\* Robert M. Girvin Fellow in Research Medicine.

either 7–11°C. or 33–38°C. At each bleeding the animals were removed to a table, cloacal temperature measured, and about 20 cc. of blood removed under oil by puncture of the heart, the needle being introduced in the midline at the junction of four scales and just caudal to the end of the sternum. The blood was defibrinated, centrifuged, and the serum separated under oil. The coagulation time was very slow in animals kept at low temperature and the clot often failed to collect on the defibrinating rod so that the serum had to be centrifuged off of partially clotted blood. The serum showed no hemolysis except in one experiment, when it was slight. In high temperature experiments the defibrination proceeded as in mammals.

The activity of the animals varied with the temperature at which they had been kept. At the low temperature they were sluggish and offered little or no resistance to handling. At the high temperature they were extremely active, combative animals, and struggled on being handled.

*Protein Refractive Index.*—The refractive index of the serum was determined at 17.5° with the Abbé refractometer and calculated according to Reiss as follows:

$$[(I_s - I_w) - 0.0028] \div 0.00172 = \text{gm. Pr per 100 cc.} \quad (1)$$

where  $I_s$  = refractive index of serum

$I_w$  = refractive index of water

0.0028 = arbitrary correction for salts and non-electrolytes.

*Kjeldahl.*—Total nitrogen was determined by the micro-Kjeldahl method of Van Slyke (2) and converted from N to protein by the factor 6.25.

*Conductivity* (3) was determined with the ionometer and *corrected conductivity* calculated as follows:

$$C_c = C_o \left( \frac{100}{100 - 2.2 [\text{Pr}]} \right) 1.22 \quad (2)$$

where  $C_o$  = corrected conductivity

$C_o$  = observed conductivity expressed as mm per liter of NaCl in aqueous solution required to give the same conductivity

$[\text{Pr}]$  = protein in gm. per 100 cc.

2.2 = factor of Gram and Cullen (3)

1.22 = arbitrary constant which brings the average of

all  $C_b$  values equal to the average of all total base values in this study.

*Total base, phosphates, and non-protein nitrogen* were determined on a 1:10 trichloroacetic acid filtrate from the serum by the methods of Stadie and Ross (4) for base, Benedict and Theis (5) for phosphates, and Van Slyke (2) micro-Kjeldahl, respectively.

*Chlorides* were determined by the method of Van Slyke (6).

*Glucose and lactic acid* were determined on a phosphotungstic acid filtrate by the methods of Folin (7) and of Clausen (8) respectively.

*pH* was determined by Hastings and Sendroy's (9) modification of Cullen's colorimetric method at the same temperature as the cloacal temperature of the animals, the empirical correction being determined by comparison of colorimetric and gasometric pH at both 9 and 38° on several samples. This correction was found to be  $+0.05 \pm 0.04$  and to all colorimetric readings 0.05 was added.

### Calculations.

*Averaged values* of Table II were obtained from Table I; the values for December 10 and 20 being the values obtained from the pooled serum of the four alligators, Nos. 2, 3, 4, and 5, were given a fourfold weight in computing the averages.

*pK'*.—The variation of  $pK'_{[H_2CO_3]}$  with temperature was taken from Cullen, Keeler, and Robinson (10):

$$pK'_{t^{\circ}} = 6.10 + 0.0050 (38^{\circ} - t^{\circ}) \quad (3)$$

Since the alligator serum has about the same ionic strength as mammalian serum, this is a sufficiently accurate approximation.

$[BHCO_3]$ ,  $P_{CO_2}$ .—These values were calculated from the equation

$$pH = pK' + \log \frac{[CO_2] - \alpha_{PCO_2}}{\alpha_{PCO_2}} \quad (4)$$

and

$$[BHCO_3] = [CO_2] - \alpha_{PCO_2} \quad (5)$$



TABLE

## Data in De

Date.....	Low temperature.						High temperature.				
	Oct. 8		Oct. 25				Oct. 15				Dec. 10.
	2	3	2	3	4	5	2	3	4	5	Pooled.
Animal.....											
Rectal temperature, °C.....	9.5	9.8	8.5	8.5	8.5	8.5	34.6	35.8	34.8	36.0	35.0
Protein refractive index, gm. per 100 cc.....	4.36	5.29	4.83	4.77	4.94	5.06	5.23	5.29	5.18	5.81	5.75
Kjeldahl, gm. per 100 cc.....	4.44	4.83					5.11	5.25	5.02	5.82	5.84
Corrected conductivity, m.-eq.....	162	170	165	161	163	162	166	171	166	175	155
Total base, m.-eq.....	168		164				171	162		167	144
Freezing point Δ, °C.....	0.54	0.56					0.57				
Cl, m.-eq.....	106.2		107.6				103.3	107.9	108.6	111.8	108.5
CO <sub>2</sub> , mm.....	27.7		24.6	25.2	25.0	25.3	23.9	15.8	15.1	13.3	15.6
pH, colorimetric.....	7.72		7.72	7.65	7.71	7.68	7.75	7.23	7.19	7.26	7.30
Lactic acid, m.-eq.....							1.0				13.0
PO <sub>4</sub> , mm.....							0.26				0.52
Glucose, mg. per 100 cc.....						66.6	115				193
Non-protein N, mg. per 100 cc.....	17.9						19.1				

TABLE II.  
Averaged Values.

Observed.	Low t° = 9 °C.		High t° = 35 °C.	
	No. of bleedings.	Value.	Value.	No. of bleedings.
Temperature, cloacal, °C.....	10	9	35	8
Protein refractive index, gm. per 100 cc.....	10	4.83	5.56	8
Kjeldahl, gm. per 100 cc.....	6	4.80	5.57	8
Corrected conductivity, m.-eq.....	10	162.7	162.3	8
Total base, m.-eq.....	10	162.4	153.7	7
Cl, m.-eq.....	10	105.6	109.3	8
CO <sub>2</sub> , mm.....	9	24.8	15.6	8
pH, colorimetric.....	9	7.72	7.27	8
Lactic acid, m.-eq.....	4	1.0	13.0	4
PO <sub>4</sub> , mm.....	4	0.26	0.52	4
Glucose, mm.....	5	5.8	10.7	4
Calculated.				
pK'.....		6.25	6.12	
BHCO <sub>3</sub> , m.-eq.....	9	24.0	14.6	8
$\alpha\text{CO}_2 \div (760 \times 2.24)$ .....		0.0709	0.0327	
Pco <sub>2</sub> , mm. Hg.....	9	12	32	8
H <sub>2</sub> PO <sub>4</sub> ' + HPO <sub>4</sub> '', m.-eq.....	4	0.49	0.91	4
BPr, m.-eq.....	9	9.2	10.6	8
BCl + BHCO <sub>3</sub> + BH <sub>2</sub> PO <sub>4</sub> + B <sub>2</sub> HPO <sub>4</sub> + BPr + B · C <sub>3</sub> H <sub>5</sub> O <sub>3</sub> , m.-eq.....	4	140.3	148.4	4
Residual B, m.-eq.....	4	22.3	10.6	4
pH - pI.....		2.40	2.42	
pK <sub>water</sub> .....		14.45	13.70	
pOH = pK <sub>w</sub> - pH.....		6.73	6.43	
$-\log \frac{\alpha_{\text{H}^+}}{\alpha_{\text{OH}^-}} = \text{pH} - \text{pOH}$ .....		0.99	0.84	

[BPr].—This value is calculated from an equation of the form of Equation 54 of Van Slyke, Wu, and McLean (11).

$$[\text{BPr}] = k\text{Pr}(\text{pH} - \text{pI}) \quad (6)$$

where [BPr] = base bound by protein in m.-eq. per liter

[Pr] = protein in gm. per 100 cc.

pI = the pH obtained by extrapolating the straight line relationship of [BPr] against pH to [BPr] = 0.

k and pI were evaluated as follows: Pooled alligator serum was saturated at two CO<sub>2</sub> tensions at both 7 and 38°C. Total CO<sub>2</sub>, CO<sub>2</sub> tension, and protein concentration were determined on the equilibrated serum and [BHCO<sub>3</sub>] and gasometric pH calculated at both temperatures by Equations 3, 4, and 5.

We found

$$\frac{\Delta [\text{BHCO}_3]}{\Delta \text{pH}} = k[\text{Pr}] = 0.79 [\text{Pr}] \quad (7)$$

Comparison of pH values at constant [BHCO<sub>3</sub>] at the two temperatures gave

$$\frac{\Delta \text{pH}}{\Delta t^\circ} = -0.018 \quad (8)$$

which is the change of pH at constant [BHCO<sub>3</sub>] or the change of pI per °C. of temperature change.

Therefore

$$\text{pI}_{t^\circ} - \text{pI}_{38^\circ} = 0.018 (38^\circ - t^\circ) \quad (9)$$

For our purpose the change of pI with temperature is important but the absolute value of pI at any temperature of relatively less importance. We, therefore, assumed for pI<sub>38°</sub> the same value found by Van Slyke, Wu, and McLean for horse serum at 38°, namely 4.80. Substituting these values for k from Equation 7 and for pI from Equation 9 and the value of 4.80 at 38° into Equation 6 we obtain

$$[\text{BPr}] = 0.79[\text{Pr}] (\text{pH} - \text{pI}) = 0.79[\text{Pr}] (\text{pH} - [4.80 + 0.018 (38^\circ - t^\circ)]) \quad (10)$$

*Residual Base.*—This term is used to indicate base not bound by chloride, bicarbonate, phosphate, protein, and lactic acid.

## RESULTS AND DISCUSSION.

The results on the four animals at any one temperature on the same or different days were so satisfactorily consistent as to justify the averaging of the data at each temperature as shown in Table II. The calculated values have been calculated from these averaged values.

The changes observed with increased temperature are as follows: There is marked increase in the concentration of glucose and lactic acid in the serum, and slight increase in the protein, phosphate, and chloride. Whether these changes have any relation to the greatly increased activity at higher temperature we do not know but it is interesting to note the association.

TABLE III.  
*Distribution of Anions Binding Base. Averaged Values.*

$t^{\circ}$		9 °C.	35 °C.
		<i>m.-eq.</i>	<i>m.-eq.</i>
Total base.....		162.6	158.4
Increased with temperature.	{ BCl.....	105.6	109.3
	{ $\text{BH}_2\text{PO}_4 + \text{B}_2\text{HPO}_4$ .....	0.5	0.9
	{ BPr.....	9.2	10.6
	{ $\text{B} \cdot \text{C}_3\text{H}_5\text{O}_8$ .....	1.0	13.0
Decreased with $t^{\circ}$ .	{ $\text{BHCO}_3$ .....	24.0	14.6
	{ B, residual.....	22.3	10.6

With rise in temperature the  $[\text{BHCO}_3]$  and the residual base decrease and possibly also the total base (see Table III).

The decrease in serum pH with rise in temperature is perhaps the most interesting finding. This change with temperature is found to be practically the same as the change in pH required to maintain constant  $[\text{BPr}]$  in a given serum with changing temperature. It has been shown by Stadie, Austin, and Robinson (12) that for either serum or whole blood the change in pH with temperature which maintains base bound by protein constant is the same and is  $\frac{\Delta\text{pH}}{\Delta t^{\circ}} = -0.017$  to  $-0.023$ . Our determination on pooled alligator serum gave a value of  $-0.018$ . In the alligator

apparently the change in pH occurring in the living animal with change in temperature is approximately such as to maintain (pH - pI) and hence the base bound by protein in the serum and cells constant; for over physiological ranges of pH

$$\text{pH} - \text{pI} = \frac{[\text{BPr}]}{k[\text{Pr}]}$$

With changing temperature, therefore, in the poikilothermous animal we observe change in pH, pOH, and  $\frac{\alpha_{\text{H}^+}}{\alpha_{\text{OH}^-}}$  but constancy of pH - pI.

That with change in temperature the change in pH should be such as to keep the base bound by protein constant is probably of fundamental importance, for the osmotic pressure across each cell membrane is affected by the base bound by protein. Any general shift in base bound by protein would lead to redistribution of the relative water content of the various cells and tissues. This relationship was pointed out for blood at any one temperature by Van Slyke, Wu, and McLean in Table II and Fig. 2 of their paper. In the table and figure cited they give relative cell volume against pH of serum. To make the relationships valid at different temperatures one must substitute for pH, the values of pH - pI.

It cannot be expected that the homothermous animal will exhibit the same change in its acid-base equilibrium in response to change in temperature as the poikilothermous animal. In fact, there are observations in the literature of interest in this connection in comparison with our results just described. Fall in temperature in the normal mammal is followed by rise of metabolism possibly accompanied by shivering. Geiger (13) reports that chilling rabbits and dogs sufficiently to cause fall in their body temperature leads to hyperglycemia which disappears as the body temperature is restored to normal. Exposure of man to hot baths leads, as shown by Koehler (14), to hyperventilation, fall of  $[\text{CO}_2]$ , and rise of pH.

Each of these effects in mammals is in the opposite direction to the change observed with change of temperature in the alligator and presumably the change observed in mammals is to be attributed to the regulatory mechanisms which maintain constant body temperature while in the alligator we observe the effects of temperature *per se* on the biological functions.

## SUMMARY.

Four alligators have been studied with respect to serum composition when the external temperature has been modified so as to produce rectal temperatures of about 9 and 35° respectively.

At higher temperature there is much greater activity of the animal, marked increase in concentration of glucose and lactic acid in the serum, and slight increase of protein, phosphate, and chloride; there is a higher CO<sub>2</sub> tension. At the higher temperature there is no change or perhaps slight diminution in total base but marked diminution in BHCO<sub>3</sub> and in the residual base bound by unknown anion. At the higher temperature there is a lower pH and the change in pH is such that pH, pOH, and  $\frac{\alpha_{H^+}}{\alpha_{OH^-}}$  each change.

The change in pH with temperature is such however that the base bound by each unit of protein in the serum is maintained approximately constant. This is probably of fundamental importance in regulating the water distribution in the cells and tissues of the body.

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# FACTORS INFLUENCING THE DIFFUSIBILITY OF CALCIUM IN HUMAN BLOOD SERUM.

BY ROBERT F. LOEB AND EMILY G. NICHOLS.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

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## INTRODUCTION.

The problem of the diffusibility of serum calcium has stimulated much investigation in recent years, yet there has been little study of the factors which play an important rôle in the passage of calcium from serum, through a collodion membrane, into solutions free from proteins.

Last year, it was shown<sup>1</sup> that qualitatively, and to a certain degree quantitatively, the factors governing the diffusibility of the Ca of blood serum in a dialysis system are the same as those regulating the diffusibility of Ca in solutions of crystalline egg albumin and of serum globulin. In these experiments it was found that when the solutions were on the alkaline side of the isoelectric point of the proteins present (proteins dissociated as anions), the Ca concentration in the protein solution was greater than that in the aqueous solution, whereas when the solution lay on the acid side of the isoelectric point (proteins present as cations), the Ca concentration of the aqueous solution was greater than that of the protein solution. Furthermore, the ratio of  $\frac{[\text{Ca}] \text{ protein solution}}{[\text{Ca}] \text{ aqueous solution}}$  varied directly with the protein concentration. These facts make it evident that the Donnan equilibrium is the chief factor quantitatively in determining the diffusibility of Ca in protein solutions.

Aside from the Donnan equilibrium, another significant factor appeared to be present in view of the nature of the curves ob-

<sup>1</sup> Loeb, R. F., *J. Gen. Physiol.*, 1925-26, viii, 451.



tained for the ratio  $\frac{[\text{Ca}] \text{ protein solution}}{[\text{Ca}] \text{ aqueous solution}}$  at various hydrogen ion concentrations. Marrack and Thacker<sup>2</sup> have presented quantitative data which harmonize with this view. We believed that this additional factor consisted in the formation of Ca-protein complex ions which increased the ratio  $\frac{[\text{Ca}] \text{ protein solution}}{[\text{Ca}] \text{ aqueous solution}}$  in solutions on the alkaline side of the isoelectric point of the protein and which raised this ratio to a certain extent in more acid solutions. That such complex ions do exist seems probable from the work of Northrop and Kunitz<sup>3</sup> who have demonstrated that under certain conditions analytical and electrometric determinations of Zn show discrepancies which are more satisfactorily explained on a basis of Zn-protein complex ion formation than on a basis of changes in ionic activities. Marrack and Thacker,<sup>2</sup> as well as many others, have assumed the presence of complex calcium-protein compounds in protein solutions.

Recently, Rona and Melli<sup>4</sup> have presented experimental evidence which suggests that the addition of lecithin to collodion alters the permeability of the membranes in such a way that the ratio of diffusible to non-diffusible Ca differs from that obtained when no lecithin is added. So far as the writers are aware, there has been no confirmation of this work.

The present work was undertaken to ascertain the influence of variations in Ca and NaCl concentrations on the calcium equilibria existing between blood serum and its dialysate.

#### EXPERIMENTAL.

Blood sera were obtained from patients suffering from hypertension or cardiac decompensation and were placed in collodion sacs (10 to 12 cc. capacity) attached to rubber stoppers. Dilution effects were partially avoided by closing the openings in the stoppers with glass rods. The collodion sacs with their contents were immersed in 250 cc. of solution, the nature of which varied

<sup>2</sup> Marrack, J., and Thacker, G., *Biochem. J.*, 1926, xx, 580.

<sup>3</sup> Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924-25, vii, 25; 1925-26, ix, 351; 1925-26, viii, 317.

<sup>4</sup> Rona, P., and Melli, G., *Biochem. Z.*, 1925, clxvi, 242.

with the experiment. The dialysis time in all experiments was 18 to 20 hours. Experiments were carried on at room temperature.

The sacs were made from Merck's U. S. P. collodion. After emptying the excess of collodion from the molds, they were rotated for 2 minutes and air-dried for 4 minutes, following which the membranes were kept in water to which a small amount of thymol had been added. The sacs were always carefully tested for leaks before they were used.

Protein determinations were made by the Kjeldahl method.

Calcium determinations were made by the method of Clark and Collip.<sup>5</sup>

Chlorine was determined by the method of Van Slyke<sup>6</sup> with the addition of minor modifications. Aqueous silver nitrate was added to the solutions and allowed to stand for 20 minutes before the addition of concentrated HNO<sub>3</sub>. The tubes were then placed in the water bath.

Hydrogen ion determinations were made colorimetrically on the aqueous solutions. The determinations shown in the tables were made upon completion of dialysis.

### Results.

#### 1. Effect of Varying Calcium Concentrations on the Ratio [Ca] Serum

[Ca] Aqueous Solution -In these experiments, the sacs containing

blood sera were immersed in 250 cc. of 0.8 per cent NaCl solution the pH of which was brought to 7.4 by the addition of NaHCO<sub>3</sub>. Calcium chloride was added to this solution so that the Ca content varied from about 0.3 to 3.0 millimols per liter (in different bottles). Table I shows that at equilibrium, the ratio of [Ca] in the sac containing serum to [Ca] in the outside solution varies strikingly with the amount of Ca present and that the smaller the amount of CaCl<sub>2</sub> added to the outside fluid, the greater is the ratio  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$ . While the Ca ratio shows this

marked change with varying concentrations of calcium, the

<sup>5</sup> Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, lxiii, 461.

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1923-24, lviii, 523.

TABLE I.

*Effect of Varying Calcium Concentrations on Diffusibility of Serum Calcium.*

Serum No. (1)	Calculated [Ca] serum. (2)	Deviation from observed [Ca] serum.* (3)	Observed [Ca] serum in sacs. (4)	Observed [Ca] aqueous solution. (5)	$\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}.$ (6)	[Cl] serum in sacs. (7)	[Cl] aqueous solution. (8)	$\frac{[\text{Cl}] \text{ aqueous solution}}{[\text{Cl}] \text{ serum}}.$ (9)	pH (10)	Protein. (11)	Original [Ca] of serum. (12)
	<i>mM</i>	<i>per cent</i>	<i>mM</i>	<i>mM</i>		<i>mM</i>	<i>mM</i>			<i>per cent</i>	<i>mM</i>
1	0.90	69	1.52	0.72	<b>2.11</b>	120	134	1.12	7.1	7.9	
	0.94	68	1.58	0.75	<b>2.11</b>	119	133	1.12	7.4	7.8	
	1.71	50	2.57	1.36	<b>1.89</b>	120	134	1.12	7.4	7.6	
	1.65	49	2.45	1.31	<b>1.87</b>	119	135	1.13	6.9	8.0	
	2.46	41	3.48	1.96	<b>1.78</b>	121	135	1.12	7.5	7.9	
2	0.54	100	1.08	0.43	<b>2.51</b>	120	135	1.12	7.4	7.0	
	0.55	105	1.13	0.44	<b>2.57</b>	120	135	1.12	7.4	6.8	
	0.94	71	1.63	0.75	<b>2.17</b>	121	135	1.11	7.4	7.0	
	0.99	69	1.67	0.79	<b>2.11</b>	120	135	1.12	7.4	7.0	
	1.87	43	2.68	1.49	<b>1.79</b>	123	136	1.11	7.4	6.7	
	1.82	44	2.62	1.45	<b>1.81</b>	123	136	1.11	7.4	7.0	
3	0.49	106	0.91	0.39	<b>2.33</b>	121	136	1.12	7.1	7.3	
	0.90	63	1.47	0.72	<b>2.04</b>	121	135	1.12	7.1	7.0	
	1.76	39	2.45	1.40	<b>1.75</b>	121	136	1.12	7.1	7.0	
	2.58	29	3.34	2.06	<b>1.62</b>	122	136	1.11	7.4	6.8	
	3.26	27	4.13	2.60	<b>1.59</b>	123	138	1.12	7.2	7.0	
	3.25	31	4.25	2.59	<b>1.64</b>	123	136	1.11	7.1	7.3	
4	0.57	48	0.85	0.48	<b>1.77</b>	125	136	1.09	6.9	6.2	2.35
	0.52	69	0.88	0.43	<b>2.05</b>	124	137	1.10	6.9	6.3	2.35
	0.81	53	1.24	0.67	<b>1.82</b>	125	138	1.10	7.1	6.1	2.35
	1.65	28	2.12	1.36	<b>1.56</b>	125	137	1.10	6.8	6.1	2.35
	1.62	33	2.15	1.34	<b>1.57</b>	126	138	1.10	6.8	6.3	2.35
	2.21	14	2.75	1.93	<b>1.43</b>	127	136	1.07	6.9	6.0	2.35

\*  $\frac{\text{Observed [Ca] serum} - \text{calculated [Ca] serum}}{\text{Calculated [Ca] serum}} \times 100.$

ratio  $\frac{[\text{Cl}] \text{ aqueous solution}}{[\text{Cl}] \text{ serum}}$  remains constant. Not only does the ratio  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$  increase with decreasing concen-

TABLE II.

*Effect of Varying NaCl Concentrations on Diffusibility of Serum Calcium.*

Serum No. (1)	[Ca] serum in sacs. (2)	[Ca] aqueous solution. (3)	$\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$ (4)	[Cl] serum in sacs. (5)	[Cl] aqueous solution. (6)	$\frac{[\text{Cl}] \text{ aqueous solution}}{[\text{Cl}] \text{ serum}}$ (7)	pH (8)	Protein. (9)	Original [Ca] serum. (10)
	<i>mM</i>	<i>mM</i>		<i>mM</i>	<i>mM</i>			<i>per cent</i>	<i>mM</i>
5	3.14	1.28	<b>2.45</b>	36.4	41.3	1.13	7.1	6.3	
	2.56	1.31	<b>1.95</b>	64.8	70.9	1.09	7.1	5.9	
	2.30	1.40	<b>1.64</b>	95.9	104.0	1.08	6.9	5.9	
6	3.32	1.25	<b>2.66</b>	20.3	25.1	1.24	7.0	6.8	2.52
	3.05	1.18	<b>2.59</b>	33.2	40.2	1.21	7.0	6.7	2.52
	2.48	1.27	<b>1.95</b>	64.6	72.1	1.12	6.9	6.8	2.52
	2.15	1.31	<b>1.64</b>	94.2	103.0	1.09	6.8	6.7	2.52
	2.00	1.33	<b>1.51</b>	127.0	138.0	1.09	7.2	6.8	2.52
7	3.68	1.21	<b>3.04</b>	19.8	24.1	1.22	7.2	8.2	2.70
	3.19	1.27	<b>2.51</b>	34.7	45.5	1.31	7.1	8.4	2.70
	2.85	1.26	<b>2.26</b>	65.4	72.3	1.11	7.1	8.4	2.70
	2.56	1.27	<b>2.01</b>	94.5	106.0	1.12	7.0	8.3	2.70
	2.37	1.31	<b>1.81</b>	124.0	139.0	1.12	7.1	8.4	2.70
8	3.20	1.22	<b>2.62</b>	19.7	24.0	1.22	7.1	5.3	2.1
	2.98	1.20	<b>2.48</b>	35.0	38.5	1.10	7.3	5.0	2.1
	2.35	1.23	<b>1.91</b>	65.0	71.2	1.10	7.1	5.2	2.1
	2.08	1.34	<b>1.55</b>	97.9	103.0	1.05	7.0	5.4	2.1
	1.98	1.28	<b>1.55</b>	128.0	137.0	1.07	6.9	5.3	2.1

trations of Ca in the dialysis system, but a concomitant change in the per cent of deviation of the theoretical Ca concentration of the serum from the observed values is apparent. As the Ca ratio decreases there is a decrease in the per cent variation from the theoretical Donnan value (Table I, Columns 2 and 3). The

theoretical [Ca] of the serum in the sac was calculated by substitution of the observed [Ca] of the aqueous solution in the equation

$$r = \frac{[\text{Cl}] \text{ aqueous solution}}{[\text{Cl}] \text{ serum}} = \frac{\sqrt{[\text{Ca}] \text{ serum}}}{\sqrt{[\text{Ca}] \text{ aqueous solution}}}$$

where  $r$  = the Donnan ratio.

In these calculations, the water content of the protein and aqueous solutions has been disregarded in order to make the theoretical [Ca] of the inside solution (serum) comparable with the observed values.

2. *Effect of Varying NaCl Concentrations on the Ratio  $\frac{[\text{Ca}] \text{ Serum}}{[\text{Ca}] \text{ Aqueous Solution}}$* .—In these experiments, the 250 cc. of solution in which the sacs containing sera were immersed were composed of NaCl, of concentrations varying between about 20 to 135 millimols per liter. The Ca concentration in all of the outside solutions, which were brought to pH 7.4 with  $\text{NaHCO}_3$  was 1.45 millimols per liter at the beginning of dialysis. Table II shows that the concentration of NaCl is important in so far as the relationship of [Ca] in the serum to [Ca] in the dialysate is concerned. A decrease in NaCl concentration increases the ratio  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$ . In those experiments in which the concentrations of NaCl were as low as 40 millimols per liter there was usually an increase in the ratio  $\frac{[\text{Cl}] \text{ aqueous solution}}{[\text{Cl}] \text{ serum}}$ .

#### DISCUSSION.

From the results presented above it is obvious that in studies on the diffusibility of calcium, not only must the concentration of protein and the pH be controlled (determining the Donnan equilibrium), but the actual concentrations of Ca and of NaCl must also be considered as they have been shown to be significant variables. The fact that low concentrations of Ca in the dialysis system increase the ratio  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$  while higher concentrations of Ca decrease this ratio strongly suggests that

part of the Ca of the serum is present as Ca-protein complex ion. When small amounts of Ca are present in the system, the ratio

$\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$  is high because apparently most of the

Ca available is required for the formation of the Ca-protein complex ions. With higher concentrations of Ca, the proteins appear to become more nearly saturated and the excess Ca is distributed in accordance with the Donnan law, the ratio thus being lowered. The saturation of the protein to form Ca-protein complex ions appears to be incomplete in view of the fact that the discrepancy between theoretical and observed Ca of the serum (Table I, Columns 2 and 4) increases somewhat with increasing concentrations of Ca in the dialysis system.

Some years ago, one of the writers<sup>7</sup> found that when serum is dialyzed against large amounts of distilled water at pH 7.4, about 25 to 45 per cent of the Ca failed to dialyze through a collodion sac, whereas when the serum was dialyzed against large amounts of 0.6 per cent NaCl at the same pH, all of the Ca diffused through the membrane. It was suggested at the time that the lack of diffusibility of serum Ca against distilled water at pH 7.4 was due to the precipitation of Ca proteinate. The experiments recorded in the present paper show that the ratio

$\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$  increases with decreasing NaCl concentration. This fact also is probably the result of precipitation of Ca proteinate.

#### CONCLUSIONS.

1. The concentration of calcium in a dialysis system is an important factor in determining the ratio of  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$ . The smaller the amount of calcium present, the greater is the ratio.

2. The concentration of NaCl in a dialysis system is significant in determining the value of the ratio  $\frac{[\text{Ca}] \text{ serum}}{[\text{Ca}] \text{ aqueous solution}}$ . The lower the concentration of NaCl, the greater is the ratio.

<sup>7</sup> Loeb, R. F., *J. Gen. Physiol.*, 1923-24, vi, 453.

3. It is obvious that in studying the diffusibility of calcium in disease conditions by means of the dialysis method, the Ca concentrations must be comparable in different experiments, the NaCl content must be constant, and the protein concentration as well as the pH must be controlled.

## THE FAT-SOLUBLE VITAMIN CONTENT OF HEN'S EGG YOLK AS AFFECTED BY THE RATION AND MAN- AGEMENT OF THE LAYERS.\*

By R. M. BETHKE, D. C. KENNARD, AND H. L. SASSAMAN.

*(From the Department of Animal Industry, Ohio Agricultural Experiment  
Station, Wooster.)*

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The value of a food product as a source of vitamins in the human dietary is largely determined by other inherent factors. Of two or more substances having a similar vitamin content the one which is the most available at a reasonable price to the greatest number of people, and at the same time is a product which is palatable, easily digestible, and possesses other food values, is the one which will finally prove the more important. In respect to these considerations hen's eggs probably take first place when compared with other natural food products as a source of vitamins A and D.

That egg yolk is a rich source of vitamin A was pointed out by McCollum and Davis (1) and by Osborne and Mendel (2) in their early work on this vitamin. More recently Murphy and Jones (3) working on the vitamin A content of fresh eggs found that about 0.25 gm. of whole egg was required daily to cure rats of xerophthalmia, and 0.5 to 0.75 gm. daily to restore normal weight.

Working with the yolk of fresh eggs, Hess (4) found that 0.25 gm. daily proved sufficient to protect rats from rickets. Similar results were obtained by Casparis, Shipley, and Kramer (5). Work at the Ohio station (6) also demonstrated that egg yolk possessed distinct antirachitic properties in preventing leg weakness in young growing chicks. Hart, Steenbock, and coworkers (7) in studying the effect of ultra-violet light on production, hatchability, and fertility of the egg, found that egg yolk from irradiated

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hens was approximately 10 times as potent in antirachitic properties as egg yolk from non-irradiated hens. Similar observations were reported by Hughes, Payne, and Latshaw (8).

The fact that milk very often is deficient in iron and the antirachitic vitamin has led many pediatricians to incorporate fresh egg yolk in the diet of the infant. Hess (4) reported that for 6 months about 50 babies were fed with excellent results a mixture consisting of milk (24 ounces), barley water (12 ounces), sugar ( $\frac{1}{4}$  ounce), and 1 egg yolk. He further states that egg yolk is well tolerated by babies and that it "possesses marked antirachitic properties . . . far more than any other natural food product."

The increasing use of eggs in the human dietary, especially for infants and invalids, makes it imperative that we have further information as to what extent the vitamin A and D content of hen's egg may be affected by the ration and management of the respective laying flocks.

#### EXPERIMENTAL.

For this study eggs were gathered from several groups of White Leghorn hens which had been under the same practical management and had received the same basal ration supplemented in various ways for 9 months. The basal ration fed these hens was a dry mash composed of ground yellow corn 30 parts, ground wheat 20, ground oats 20, wheat middlings 10, wheat bran 10, meat scraps 10, and oyster shells and water *ad libitum*. No scratch grain was fed. Pen 12 was confined indoors and received the basal mash fortified with 2 parts cod liver oil. Pen 13 received the basal ration only and was confined indoors. Likewise Pen 15 was kept indoors and fed the basal mash plus chopped alfalfa hay *ad libitum*. Pen 16 received the same mash but had access to a blue-grass range. The only sunlight available to Pens 12, 13, and 15 was that which filtered through the closed windows on the southern exposure.

#### Vitamin A.

For the determination of vitamin A, 12 litters, of six each, of young healthy stock rats, 23 to 24 days of age and weighing from 45 to 60 gm., were confined in our standard laboratory cages and

fed a basal ration devoid of vitamin A. The ration had the following composition: casein 18 parts, starch 71, Crisco 5, salt mixture 4, and agar 2. Vitamin B was supplied by 0.25 gm. of a commercial yeast fed separately each day. All animals were irradiated 10 minutes, every other day, at a distance of 30 inches with light from a quartz mercury vapor lamp.

The casein was prepared by extracting the commercial product repeatedly with alcohol and ether for 5 days and then drying at 110–120°C. for 24 hours. Air was passed through the Crisco for 24 hours at 100°C. to insure the destruction of any vitamin A which may have been present. The starch was not specially treated, since it was not found to contain a detectable quantity of this vitamin. The salt mixture employed was that described by McCollum (9) with the addition of 0.25 per cent potassium iodide.

Preliminary experiments with young rats of our stock colony showed that the vitamin A reserves of their bodies were depleted between the 4th and 5th week. Accordingly, at the close of the 5th week the rats from the various litters were confined in individual cages and distributed so that no two animals from the same litter received the same quantity of any particular kind of egg yolk. They were continued on the same basal ration with 0.25 gm. of yeast fed separately each day and irradiated as before. Varying amounts of egg yolk diluted 1:1 from the four different lots of hens (designated as Egg Yolk 12, 13, 15, and 16 to correspond with the individual pen treatment) were fed separately each day for 7 weeks.

The egg yolks were prepared by carefully separating the yolk from the white of 2 dozen eggs and then adding an equal weight of distilled water and mixing thoroughly with an egg beater. The diluted yolks were then transferred to stoppered flasks and stored at 34°F. Fresh quantities were prepared every 2 weeks.

The results are recorded in Charts I and II. Chart I shows how young rats fed the vitamin A-deficient ration rapidly declined in weight after the 5th week with the development of xerophthalmia, and respiratory troubles between the 6th and 7th week. The feeding of increasing amounts of the diluted yolks from Pens 13 and 15 caused a progressive delay in the development of xerophthalmia with a prolongation of life and increased resumption of growth.

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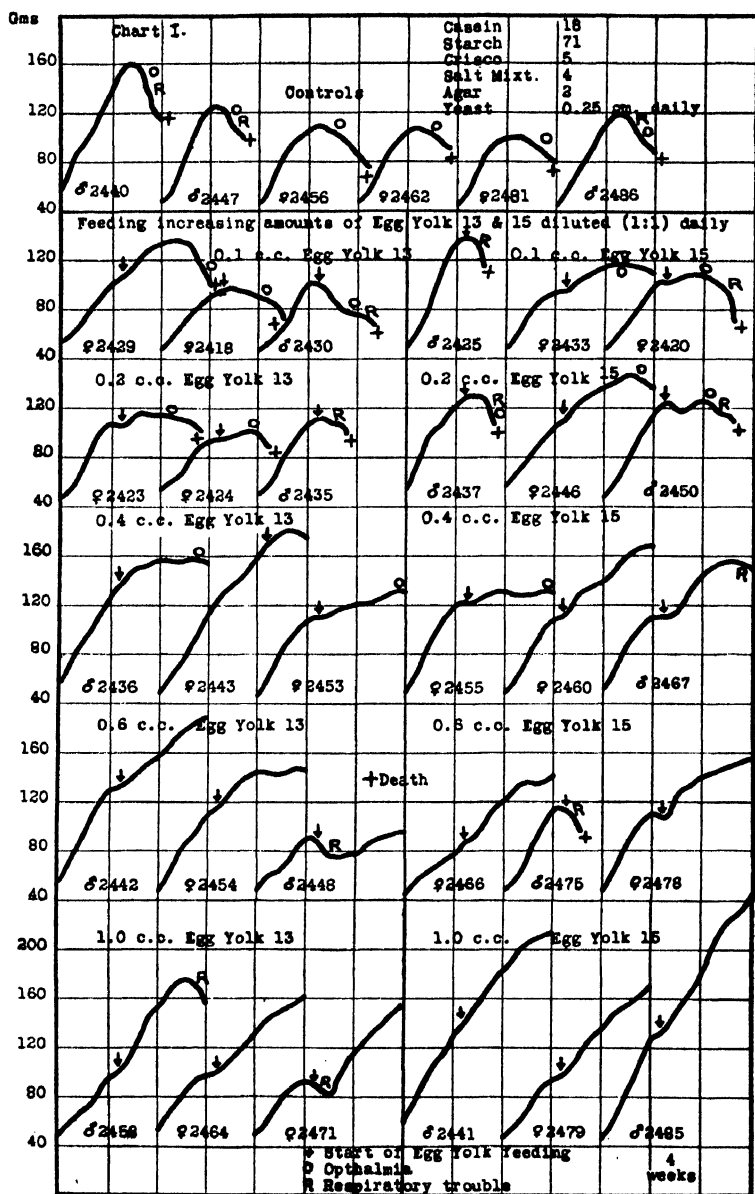


CHART I.

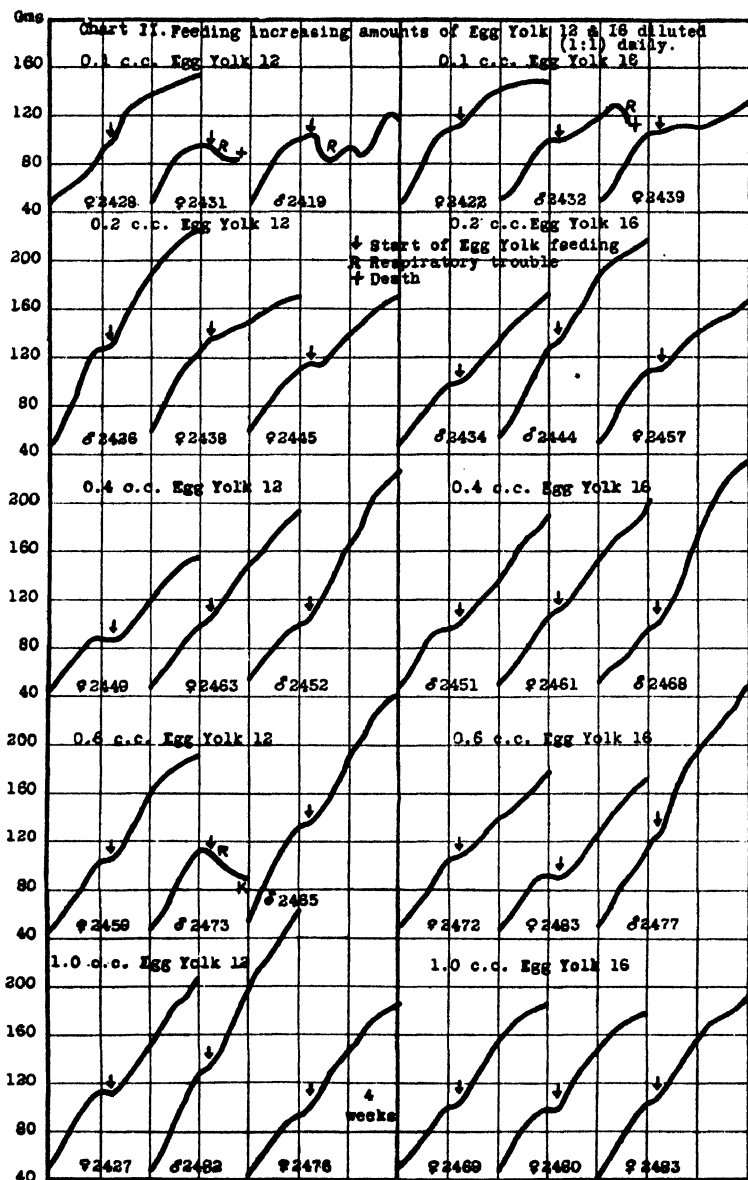


CHART II.

## 700 Fat-Soluble Vitamin Content of Egg Yolk

The comparative growth curves show no substantial difference in the vitamin A potency of the egg yolks from Pens 13 and 15, although the yolks from Pen 15 appear to be the better of the two in that apparently normal resumption of growth and behavior were obtained when 1.0 cc. of the diluted yolk material was fed daily, while an equivalent quantity of yolk from the basal pen (No. 13) caused slower gains with evidence of respiratory troubles.

Chart II shows the effect of feeding increasing amounts of diluted egg yolks from the cod liver oil (No. 12) and blue-grass range (No. 16) pens. Both of these yolk materials were considerably more potent in vitamin A than those from Pens 13 and 15. In comparing the growth curves of Charts I and II it is quite evident that eggs from Pens 12 and 16 were approximately 5 times more potent than yolks from Pens 13 and 15. Only 0.2 cc. of the diluted yolk from Pens 12 and 16 was required to produce apparently normal behavior in contrast to 1.0 cc. of egg yolk from Pen 15 and more than 1.0 cc. from the basal group (Pen 13). Rat 2473 which received 0.6 cc. of the diluted egg yolk from Pen 12 developed a severe respiratory trouble 2 days after egg yolk feeding was begun. This was followed by what appeared to be an infection of the brain from which the animal did not recover and consequently was killed after the 8th week.

No attempts were made to determine the comparative vitamin A content of the rations fed the hens. From work published by numerous investigators it is evident that the basal ration was low in this factor and was correspondingly low in the egg. The addition of alfalfa hay, fresh green grass, and cod liver oil to the dietary of a laying hen increased the vitamin A intake of the bird and accordingly the potency of the egg yolk. The alfalfa hay used in this trial was a commercial product and not of the best grade, which may in part account for the small difference in the vitamin A content of the eggs from Pens 13 and 15. Another interesting point is the fivefold increased vitamin content of the eggs from the pen on blue-grass range (Pen 16), in contrast to the alfalfa hay group—suggesting that fresh green grass is more potent in vitamin A than the product made into hay with subsequent exposure to variable weather conditions.

*Vitamin D.*

To determine the comparative antirachitic properties of the eggs from the variously treated groups of hens, young rats were fed the egg yolks at levels ranging from 0.5 to 7 parts added to a rickets-producing ration and then examined for the severity of the rickets produced. The rats used were raised in our laboratory under standardized conditions. These rats, weighing from 45 to 60 gm., were taken at an age of 24 to 25 days and distributed in groups of three according to litter and sex.

The rickets-producing ration employed was the one reported by Steenbock (10), consisting of yellow corn 76 parts, wheat gluten 20, calcium carbonate 3, sodium chloride 1. In our experience this ration has proved very satisfactory, producing incipient rickets in our young stock rats in a few weeks and very severe rickets in 4 to 5 weeks. The egg yolk was added to the rachitic ration in varying parts per hundred.

In preparing the yolks, 3 dozen eggs from a particular pen were taken, broken individually, and the yolk separated from the white as carefully as possible. All the yolks from a certain pen were then placed in a beaker, twice the weight of distilled water added, and the mixture thoroughly stirred. To 1 kilo of the ration was then added, on an undiluted basis, 0.5 to 7.0 gm. of the egg yolk. After thoroughly mixing the yolk material with the ration the mixture was dried for 36 hours at 65°C. and then ground to insure complete distribution.

After the groups had been on their respective rations for 4 weeks, note was taken of the severity of the rachitic condition in each animal as indicated by enlargement of the joints—particularly the wrists—and by general behavior, previous to autopsy. The wrists of all animals were removed and subsequently examined by silver-staining the distal ends of the ulnæ and radii after splitting with a razor blade. The femurs were also removed, freed from adhering tissue, and ashed after exhaustive extraction with hot alcohol and ether. The data are, in part, tabulated in Table I.

It is quite evident from the data secured that the yolks of the eggs from the pen fed cod liver oil (No. 12) and the blue-grass range group (No. 16) were much more antirachitically potent than those from the basal or alfalfa hay group. The rations forti-

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fied with 1.0 and 2.0 parts of the egg yolks from Pens 12 and 16, respectively, possessed greater calcifying properties than the rations carrying 5.0 parts of the yolks from groups in Pens 13 and 15. In other words, the yolks from the eggs of the range pen were more than 5 times as potent, antirachitically, as those from the basal and alfalfa hay pens. While the yolks from the cod liver oil-fed group were not as rich in the antirachitic factor as the yolks from the birds on range, they still proved to be considerably more active antirachitically than any of the other two groups.

TABLE I.

*Showing the Calcifying Properties of the Various Egg Yolks when Incorporated in a Rickets-Producing Ration.*

Egg Yolk 13 (basal pen).			Egg Yolk 12 (cod liver oil pen).		
Yolk added.	Average ash in femurs.	Metaphyses.	Yolk added.	Average ash in femurs.	Metaphyses.
gm.	per cent		gm.	per cent	
1.00	29.79	About 2 mm. wide.	0.50	27.02	About 2 mm. wide.
2.00	34.71	1.5-2 " "	1.00	33.08	1.5-2 " "
3.00	36.13	About 1.5 " "	2.00	40.91	0.5-1.0 " "
5.00	37.98	1.0-1.5 " "	3.00	43.61	About 0.5 " "
7.00	43.64	About 0.5 " "	5.00	47.69	Normal.
Egg Yolk 15 (alfalfa hay pen).			Egg Yolk 16 (outdoor range pen).		
0.50	30.89	About 2 mm. wide.	0.50	36.25	1.0-1.5 mm. wide.
1.00	32.47	1.5-2.0 " "	1.00	40.53	0.5-1.0 " "
2.00	34.01	1.5-2.0 " "	2.00	48.06	Normal.
3.00	36.85	1.0-1.5 " "	3.00	48.91	"
5.00	38.33	About 1.0 " "	5.00	57.10	"

Results represent an average of three rats per lot

Although the data in Table I show a rather convincing difference in calcifying properties between the various egg yolks, we thought it advisable to check these probable differences in another manner. For this purpose the line test method as described by McCollum and coworkers (11) was employed. In its essentials this procedure consists in inducing a rachitic condition in the growing rat by using a ration relatively high in calcium as compared to its phosphorus content. Steenbock's ration, as previously de-

TABLE II.  
*Calcium Deposition in Rachitic Rats after Feeding Egg Yolks from  
 Various Treated Hens.*

Egg Yolk 13 (basal pen).					Egg Yolk 15 (alfalfa hay pen).				
Yolk added.	Rat No.	Weight.	Average daily consumption.	Calcium deposition.	Yolk added.	Rat No.	Weight.	Average daily consumption.	Calcium deposition.
gm.		gm.	gm.		gm.		gm.	gm.	
1.00	1675	67-67	7.5	—	1.00	1727	70-75	8.5	—
	1686	75-74	8.0	—		1739	78-78	8.0	—
2.00	1676	93-103	9.5	—?	2.00	1710	82-85	7.0	+?
	1688	79-85	9.5	+		1722	74-78	8.0	+
3.00	1681	76-84	8.5	++	3.00	1728	69-74	7.5	+
	1694	73-80	8.5	++		1736	62-67	7.0	++
5.00	1682	95-105	9.0	+++	5.00	1730	65-75	7.0	+++
	1693	92-101	9.0	+++		1738	63-65	6.5	+++
Egg Yolk 12 (cod liver oil pen).					Egg Yolk 16 (outdoor range pen).				
0.20	1677	74-79	8.0	—	0.20	1717	76-81	7.5	+?
	1683	82-85	7.5	—?		1723	72-73	6.0	+
0.50	1695	83-88	8.5	+	0.50	1712	83-87	7.5	+
	1701	69-72	7.5	++		1729	71-78	8.0	++
1.00	1678	76-83	8.5	++	1.00	1724	73-73	7.5	+++
	1687	91-89	7.5	++		1731	59-63	6.5	+++
2.00	1691	71-72	7.5	+++	2.00	1713	82-88	7.5	++++
	1696	91-98	9.5	+++		1725	74-82	7.0	++++
3.00	1679	86-95	9.5	+++	3.00	1719	82-92	9.5	≠
	1707	77-81	7.0	++++		1732	68-76	8.5	≠

- No calcium deposition.  
 + Evidence of calcium deposition.  
 ++ Narrow line of calcium.  
 +++ Wide line of calcium.  
 ++++ Very wide line of calcium.  
 ≠ Union with diaphysis.



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scribed, was used. When the rats showed evidence of a rachitic condition at the end of the 3rd week they were confined in individual cages and the egg yolk from eggs of the four different groups of hens was incorporated into the ration at varying levels. The material represented a mixture of the yolks of 2 dozen eggs from each lot, prepared as previously described.

The yolk-bearing rations were fed to the rachitic rats for a 10 day period. Records were kept of their daily feed consumption. At the end of this time the rats were killed, the radii and ulnae removed and examined for calcium deposition by the silver nitrate method.

The results, in part, are summarized in Table II. The data confirm the results of the previous study (Table I); namely, that the yolks of eggs from the cod liver oil pen, No. 12, and the range group, No. 16, were very much more antirachitically potent than those from the basal pen, No. 13, or alfalfa hay pen, No. 15. The ration fortified with 0.2 part of Egg Yolk 16 (range) was as potent antirachitically as the ration fortified with 2.0 parts of Egg Yolk 13 (basal). Likewise, 0.5 part of Egg Yolk 12 (cod liver oil) proved to be better from an antirachitic standpoint than 2.0 parts of Egg Yolk 13. Further evidence is presented that the yolks of the eggs from the alfalfa hay group were no better than the yolks from the eggs of the birds receiving the basal ration only. Apparently the alfalfa hay, a commercial product, was not only low in vitamin A but also in the antirachitic factor.

### DISCUSSION.

It is clearly evident from the data available that the vitamin A and D content of egg yolk is largely, if not entirely, determined by the amount of these substances present in the ration and environment of the hen. Hart, Steenbock, and coworkers (7) as well as Hughes and coworkers (8) have shown that the amount of ultra-violet irradiation a hen receives is an important factor in determining the antirachitic vitamin content of the eggs which it produces. The results presented substantiate these findings. Cod liver oil feeding likewise increases the antirachitic as well as the fat-soluble A vitamin content of eggs. It is of interest to note that hens having access to a luxuriant blue-grass range produce eggs which possess as great or greater vitamin A and D content

than similar hens receiving the same ration fortified with 2 parts of medicinal cod liver oil. Thus, from a nutritional view-point of the egg, it would appear unnecessary and uneconomical to provide laying hens with additional vitamin A- and D-containing substances in their ration to make the eggs more potent in these factors, when they have access to a good outdoor range. Alfalfa hay, as used in this experiment, did not increase the antirachitic potency of the egg yolk and exerted but a small effect in increasing the fat-soluble A content.

The practical poultryman is at the present very much concerned with the feeding of cod liver oil and the use of ultra-violet light as they affect egg production and hatchability. Reports from the Wisconsin (7) and Kansas stations (8) indicate that hatchability is related to the antirachitic vitamin content of the egg. In this connection it is interesting to speculate why alfalfa hay, as used in this experiment, did not influence the antirachitic vitamin content of the egg yolks and yet accounted for an increase in hatchability over the basal ration. Cod liver oil, on the other hand, caused a marked increase in vitamin A and the antirachitic factor of the yolks without affecting the hatching qualities of the eggs. Apparently, other factors aside from the antirachitic and fat-soluble A vitamin content of eggs exert some influence on hatchability, etc. These relations will be discussed in a subsequent paper.

We consider it significant that our data have shown that yolks from hen's eggs vary greatly in their antirachitic and fat-soluble A vitamin content—depending upon the ration and management of the layers. The poultryman and the consumer should recognize the misconception of the idea that "an egg is an egg" in respect to both its vitamin content and its quality. An inadequate ration may yield impoverished eggs as well as animals. The true nutritional value of eggs, like that of milk, is chiefly determined by the feed and management of the flock.

The relative price of market eggs is now determined by their freshness or fulness and other physical properties. May not an additional qualification, their vitamin content, based largely on the ration and management employed with the layers, be required for the highest class of eggs in the near future? Even in the light of present information it would not be unreasonable to secure eggs for use in hospitals and for infants from flocks receiving an ade-

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quate ration and having access to a suitable outdoor range or its equivalent throughout the year.

### SUMMARY.

The fat-soluble vitamin content of hen's egg yolk is greatly influenced by the amount of these substances present in the ration and by environment of the laying hen.

Yolks of eggs laid by hens which had access to a blue-grass range were approximately 5 times as potent in vitamin A, and 10 times as active antirachitically as the yolks of eggs laid by hens which received the same basal mash but were confined indoors.

The feeding of 2 parts of cod liver oil in the mash accounted for an approximate fivefold increase in the antirachitic and fat-soluble A vitamin content of the egg yolks.

The addition of alfalfa hay to the basal mash did not improve the calcifying properties of the egg yolks, but proved of some benefit in increasing the vitamin A content.

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## STUDIES ON THE NATURE OF THE COMBINATION BETWEEN CERTAIN ACID DYES AND PROTEINS.

BY L. M. CHAPMAN, DAVID M. GREENBERG, AND CARL L. A. SCHMIDT.

*(From the Division of Biochemistry and Pharmacology, University of California Medical School, Berkeley.)*

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### INTRODUCTION.

Many investigations have shown that the phenomenon of staining of tissues and dyeing of fabrics is not one which can be expressed in terms of a simple reaction. This is due in part to the multiplex nature of the substance which takes up the stain or dye. It is further complicated by taking place in a heterogeneous system. In investigating reactions of this type it is of paramount importance to reduce, if possible, the number of variables to a minimum. As an introduction to this problem we have studied the reaction which takes place between one of the most common constituents of protoplasm, *viz.* protein, and dye. The variables have been further reduced by carrying out the reaction in a macrohomogeneous system. As a result of our studies we were led to the conclusion that the dyes and proteins which were used in our experiments react in stoichiometric proportions. The reaction between protein and dye has been used to throw further light on the behavior of proteins in solutions of high acidity.

It is not the purpose of this paper to review at length the many studies which have been directed towards the elucidation of the phenomenon of dyeing and staining. Several are, however, deserving of mention. Knecht (1) in 1907 showed that when wool is dyed in an acid solution containing the magnesium salt of crystal scarlet, all of the mineral element remains in the exhausted dye bath. Gnehm and Rotheli (2) found that when wool is dyed with the benzidine salt of naphthol yellow all of the benzidine remains in the bath. Wool is optically active. The idea was conceived by certain investigators that if the process is one of chemical combination wool will absorb two optically active isomers in unequal amounts. Willstätter (3)

tested the rate of absorption of optically isomeric alkaloids on wool and silk but found no preferential absorption. The experiments of Ingersoll and Adams (4), Porter and Ihrig (5), and Morgan and Skinner (6) show that wool removes the isomers of certain dyes from solutions in unequal amounts. The isomers of all dyes do not, however, behave alike in this respect. It has been shown that acidity is a factor which must be considered when dyes are taken up by protoplasm. Thus Irwin (7) showed that the penetration of *Nitella* cells by cresyl blue is dependent on the alkalinity of the solution, and Fleischer and Amster (8) found that the disinfecting power of certain dyes is markedly affected by slight changes in acidity. A few investigators have recognized the advantages of working with individual proteins. Thus Mathews (9) showed that the albumins precipitate basic dyes in the presence of base and acid dyes in the presence of acid. Qualitative work of a similar nature but more extensive was published by Heidenhain (10). Robertson (11) found that the distribution of a number of dyes between ethyl acetate and water is dependent on the presence of acid or alkali protein in the aqueous phase. Marston (12) has attempted the purification of the proteolytic enzymes by means of azine dyes but the specificity of the reaction is questioned by Fearon (13). The experiments of Loeb (14) probably more than of anyone else point to the conclusion that the taking up of dyes by proteins is a chemical rather than a physical phenomenon. He found that gelatin is stained by acid dyes on the acid side of the isoelectric point and basic dyes stain gelatin on the alkaline side. Grollman's work (15) on the combination of certain proteins with neutral red covers only a small portion of a curve which might be constructed to show the total capacity of proteins for this dye. On the basis of the Freundlich formula he contends that the reaction between dye and protein is an adsorption phenomenon. Robertson (16) has, however, shown that under some conditions this formula can be derived from the mass law and it is, therefore, not a true criterion for adsorption. Grollman's work indicates that the value of  $\frac{1}{n}$  in the Freundlich formula,

$$\frac{x}{m} = Kc^{\frac{1}{n}}$$

where  $x$  = dye absorbed,  $m$  = mass protein,  $c$  = equilibrium concentration, and  $K$  and  $\frac{1}{n}$  = constants, approaches very closely the value of unity. The low effective valence of proteins confirms this view (17-19). No great error will result if the value of unity be substituted for  $\frac{1}{n}$ . The Freundlich formula now expresses the mass law relationship between dye and protein.

### *Materials and Experimental Technique.*

The proteins used in the experimental work were gelatin, deaminized gelatin, casein, fibrin, and edestin. Casein was prepared

by the method of Van Slyke and Baker (20) and the Difco gelatin was purified according to the procedure of Loeb.<sup>1</sup> A modification of Bosworth's (21) method was employed in preparing fibrin, and edestin was prepared according to the method which has been described by Osborne (22) and others. Deaminized gelatin was prepared by a slight modification of Hitchcock's (23) method. It yielded no amino nitrogen. All protein preparations were iso-electric and of low ash content. Of the dyes used in the experiments Biebrich scarlet, naphthylamine brown, tropeolin O and metanil yellow were obtained from Coleman and Bell, lacmoid from Grüber, and acid violet from the National Aniline and Chemical Company. The latter substance was recrystallized. The azo and thiazine dyes were standardized by titration with  $\text{TiCl}_3$  and the triphenylmethane dyes by estimation of the nitrogen content. Dyes for which neither of these methods were applicable could be standardized against methylene blue according to the method of Pelet-Jolivet (24). Acid violet titrated as though only one acid group was reactive while Biebrich scarlet was found to be dibasic. The other dyes were monobasic. With the exception of tropeolin O none of the dyes was found to interfere with the potential measurements by means of the hydrogen electrode. Acidity measurements in solutions containing this dye were carried out by means of indicator papers, a due allowance being made for the color of the dye. The only dye which changed color to an appreciable degree in the region where it was titrated with proteins was metanil yellow. However, this had no effect on its ability to combine with protein. With the exception of tropeolin O the remaining dyes changed in depth of color with increasing acidity. The above dyes were chosen for the following reasons: (a) They are soluble in solutions of high acidity. (b) They form insoluble precipitates with the proteins used in these experiments. (c) They do not show marked color changes in the regions of acidity which were used in these experiments.

The first experiments were carried out on gelatin granules. These were placed in a solution of acid or alkali and dye was then added. After standing for various periods of time the mixture was filtered and the amount of dye left in solution was estimated

<sup>1</sup> Loeb (14), p. 41.

colorimetrically. The experiment was repeated many times with varying concentrations of gelatin and dye, the time of absorption and acidity also being varied. Typical results are shown in Figs. 1, 2, and 3. As shown in Fig. 1 time is a very important factor in the absorption of dye by gelatin granules. Because of the long time required for equilibrium it appeared impractical to use gelatin granules and further work was carried out with protein solutions. Figs. 2 and 3 show that acidity and quantity of dye added must be taken into consideration. The influence of these factors is much the same in the case of gelatin granules as in the case of protein

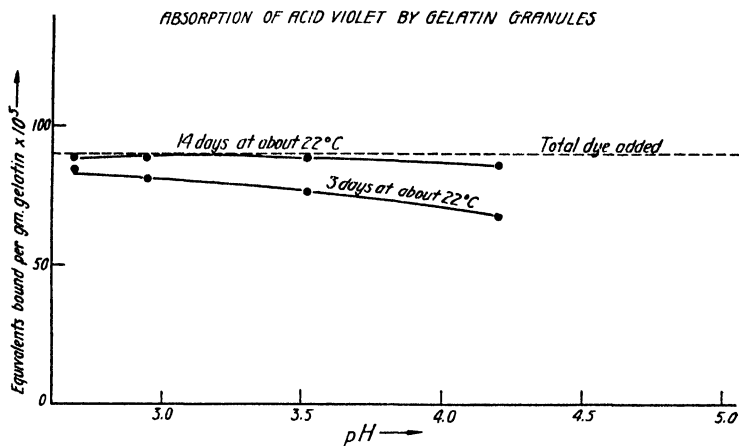


FIG. 1.

solutions and will, therefore, be treated in the discussion of protein solutions.

For the determination of the amount of dye which combines with a given amount of protein use was made of the method which was developed by Pelet-Jolivet (24) for the estimation of dyes. He found that a standardized basic dye may be used to determine the equivalence of an acid dye solution or *vice versa*, allowing the dyes to be their own indicators. The compound formed by the union of such an acid dye with a basic dye is usually insoluble. When a large excess of protein is used a protective colloidal effect is developed which prevents precipitation from taking place. As more dye is added the excess protein is neu-

tralized by the dye and the protective colloidal effect disappears. When an equivalent amount of dye has been added, if the dye-protein compound is sufficiently insoluble, the protein and dye will be quantitatively precipitated, leaving an almost colorless protein-free filtrate. When an excess of dye was added, it was never found to exert a protective colloidal effect but remained quantitatively in the filtrate while the protein was precipitated.

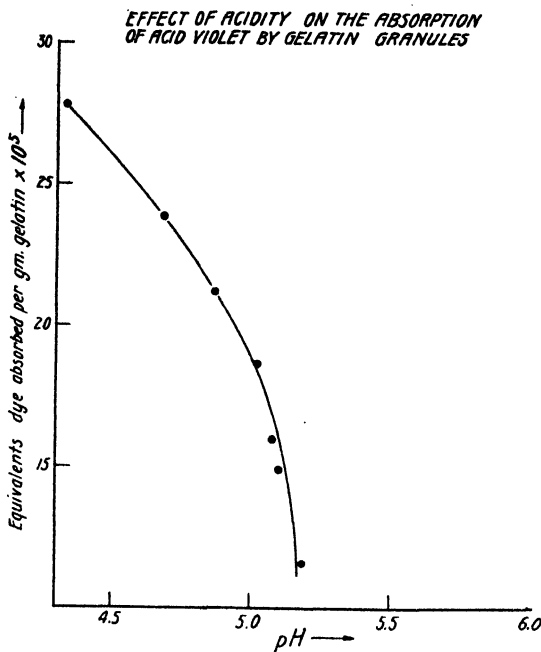


FIG. 2.

The amount of dye which was capable of combining with the protein could be determined either by titrating to an almost colorless end-point or by adding excess dye and determining that excess by colorimetric estimation of the dye in the filtrate. In the case of certain dyes (tropeolin O and metanil yellow in acidities lower than pH 1.5) no colorless end-point was obtained. In such cases only the method of using excess dye could be used. Fortunately, tropeolin O maintains a quite constant color at the acidi-



ties used. It could, therefore, be used for colorimetric comparison. On the other hand, metanil yellow changed color in the acidity region which was used, but fortunately, the excess dye which had to be added was so slight that a relatively large error in making color comparisons caused only a negligible error in the results.

With some dyes an extended region of colorless filtrates is formed instead of a sharp end-point. In such cases the last point in that zone is taken as the maximum combining capacity of that protein for the following reasons. When such dyes are used the

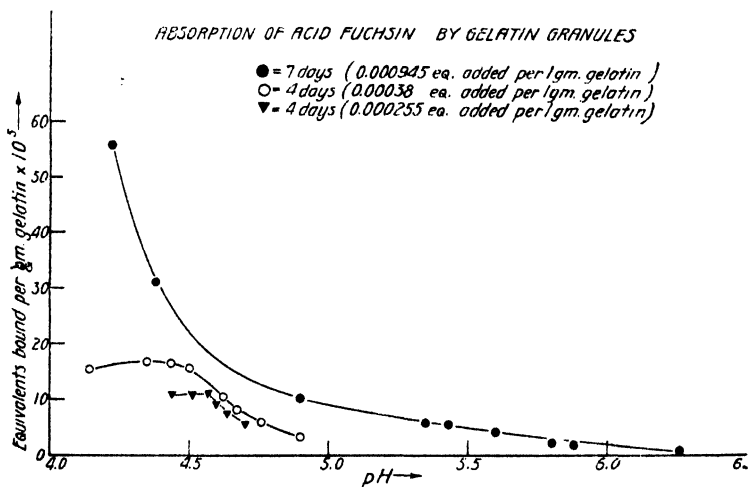


FIG. 3.

protective colloidal effect does not persist up to the end-point but ceases considerably before that point is reached. Since the protein is in excess in such cases all the dye added is precipitated until the end-point is reached. Beyond the end-point all of the protein is precipitated and the excess of dye remains in solution. Biebrich scarlet, naphthylamine brown, and acid violet have colorless regions near the end-point, and accordingly the last colorless point was taken as the end-point. In the case of Biebrich scarlet additional dye could not be added to confirm the end-point, because Biebrich scarlet is a dibasic acid dye, and an excess of dye should cause it to act more like a monobasic acid dye. Since the

filtrate was free from protein and dye at the end-point it was consequently appropriate for use. Judging from the formula, acid violet is also a dibasic acid dye. Since naphthylamine brown is a monobasic dye an excess of dye may be added and the excess determined colorimetrically. Above pH 2 the amount of naphthylamine brown which combined with gelatin at the last colorless point was found to check with the amount of naphthylamine brown which combined with gelatin when an excess dye was added as shown in Tables I and II. Consequently with this dye one

TABLE I.

*Titration of a Gelatin Solution with Naphthylamine Brown Using End-Point Method.*

pH.....	1.42	1.56	1.93	2.27	2.37
Dye precipitated by 1 gm. gelatin (equivalents).....	$106 \times 10^{-5}$	$106 \times 10^{-5}$	$104 \times 10^{-5}$	$102 \times 10^{-5}$	$99 \times 10^{-5}$

TABLE II.

*Titration of a Gelatin Solution with Naphthylamine Brown, by Adding Excess Dye and Determining That Excess Colorimetrically.*

pH.....	1.56	1.76	1.93	2.10	2.13	2.43	2.94
Dye precipitated by 1 gm. gelatin (equivalents).....	$103 \times 10^{-5}$	$104 \times 10^{-5}$	$102 \times 10^{-5}$	$103 \times 10^{-5}$	$103 \times 10^{-5}$	$105 \times 10^{-5}$	$101 \times 10^{-5}$

method of titration could be used to check the other. The remaining dyes gave sharp colorless end-points. Their titration was more simple.

In general dilute solutions are to be desired for quantitative precipitations so as to avoid as far as possible any phenomena of occlusion. When titrating, the desired amount of acid was added to the protein solution and dye was added with constant agitation. A preliminary titration was carried out to obtain the approximate end-point. In the final titration no material was removed for testing until the protein was 95 per cent titrated. This reduced

the necessary error to a minimum. On reaching the end-point the mixture was filtered and the acidity was estimated electrometrically. The tables of Schmidt and Hoagland (25) were used to interpret the electrometric measurements. In certain instances it was necessary to add considerable excess dye in order to reduce the solubility of the protein-dye compound. The excess dye in the filtrate was estimated colorimetrically. The gelatin solution used was 0.172 per cent. This solution could be kept at 10°C. without solidifying. All titrations were carried out at approximately this

TABLE III.  
*Titration of a Gelatin Solution with Tropaeolin O.*

Total dye added.	Dye precipitated by 0.086 gm. gelatin.	Remarks.
cc.	cc.	
5.2		Gelatin in filtrate.
6.5		" " "
8.5		" " "
9.5	8.58	" " "
12.0	8.70	
13.0	9.20	40 per cent excess.
14.5	9.30	
15.0	9.34	
15.5	9.20	
17.5	9.37	
17.5	9.38	
20.0	9.40	
20.0	9.40	
15.0	9.50	Added quickly, filtered immediately.
15.0	9.15	Let stand overnight before filtering.
15.0	9.60	Added the gelatin to the dye.

temperature. The concentrations of the other protein solutions were about 0.2 per cent. The proteins were brought into solution by addition of the necessary amount of acid.

### *Results.*

The results of experiments which were carried out to determine the influence of variations in the experimental conditions on the combination between dye and proteins keeping the acidity constant are shown in Table III. The amount of dye in excess of that

which was bound varied from 40 to 104 per cent. The extreme variation in the amount of dye which was precipitated from the mean value is 2.5 per cent.

The gelatin solution was titrated with six different dyes. The results are plotted in Fig. 4. For reference the acid titration curves which have been obtained by Hitchcock (26) and by Atkin and Douglas (17) are also plotted. In this and subsequent figures in addition to the titration curve a dotted line extending hori-

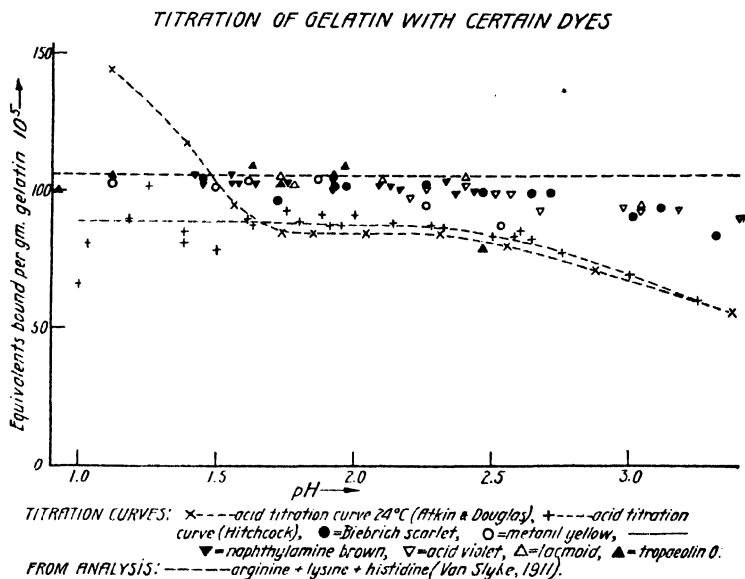


FIG. 4.

izontally across the graph has been plotted. It indicates the theoretical equivalence of the second basic groups of arginine + lysine + histidine which in the native protein molecule are probably free. Both the acid titration and the dye titration curves might be expected to fall upon this dotted line in the region of high acidity. Below pH = 2<sup>2</sup> the dyes average a titration value of about 0.00104 equivalent of dye absorbed per gm. of gelatin which is within 2.5

<sup>2</sup> This value is not absolute but is used as a convenient reference point. The titration is practically complete at this acidity.

per cent of that which would be expected from the content of hexone bases as determined by Van Slyke (27). His analysis showed 0.00107 mol per gm. of gelatin. As shown in Fig. 4 the value for the dye equivalence of gelatin is somewhat higher than the acid-binding value as given by Hitchcock (26). He found a maximum of 0.00089 equivalent of HCl bound per gm. of gelatin. Atkin and Douglas (17) found 0.00084 equivalent. The dye titration curve shows no rise analogous to that found by Atkin and Douglas (17) in the region more acid than pH 1.7.

The effect of deaminization on the acid- and dye-combining capacity of gelatin is shown in Table IV. The values are less than would be anticipated on the basis of the content of arginine +

TABLE IV.  
*Combining Capacity of Gelatin and Deaminized Gelatin.*

	Gelatin.	Deaminized gelatin.	Difference due to removal of the epsilon group of lysine.
	<i>equivalents per gm.</i>	<i>equivalents per gm.</i>	<i>equivalents per gm.</i>
Acid-combining capacity (Hitchcock)....	0.000890	0.000445	0.000445
Arginine + lysine + histidine (Van Slyke, 1911).....	0.001066	0.000661	0.000405
Dye-combining { Biebrich scarlet.....	0.001025	0.000587	0.000438
capacity. { Naphthylamine brown.	0.001030	0.000586	0.000444

histidine. The difference in the dye titration value of gelatin and deaminized gelatin is in fairly good agreement with a similar difference in the acid-combining value reported by Hitchcock (23). This is probably merely a coincidence. The fact that the dye-combining value of deaminized gelatin is less than would be expected suggests that the treatment with  $\text{HNO}_2$  has affected the gelatin to a greater extent than the removal of the epsilon amino group of gelatin. Skraup (28) and Dunn and Lewis (29) have found by analysis that the arginine and histidine content of proteins is slightly decreased on deaminization. It is also known that the guanidine group of arginine is slowly attacked by nitrous acid (30). The dye titration values are therefore not strictly quantitative but nevertheless suggestive of the combination between dye and the free amino group of lysine.

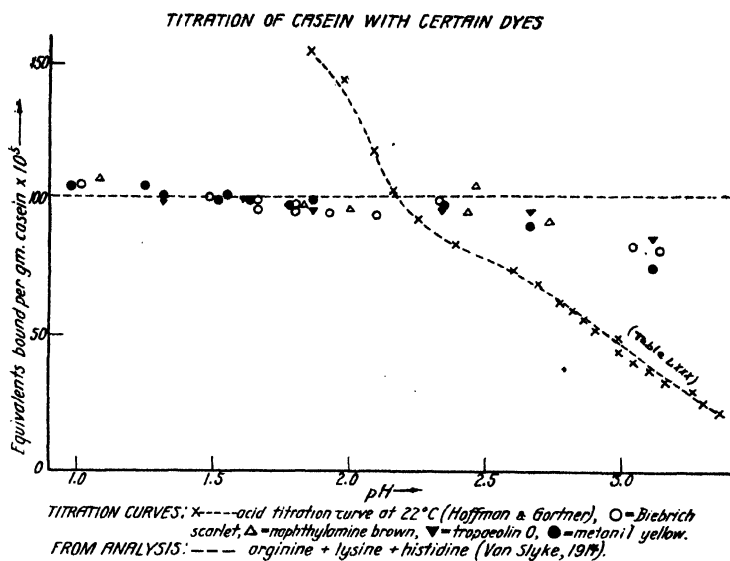


FIG. 5.

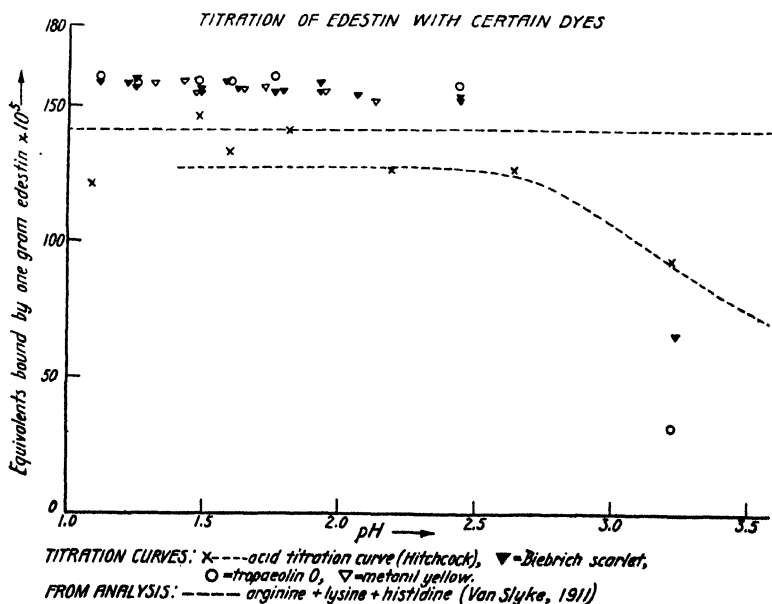


FIG. 6.

Casein was titrated with four different dyes. The curves are plotted in Fig. 5. The analysis of Van Slyke (31) was used to plot the theoretical equivalence of the second groups of the hexone bases. It indicates 0.00102 equivalent per gm. of casein. The average value for the dye titrations is 0.00100 equivalent. For comparison Hoffman and Gortner's (32) acid titration curve at 22°C. is also plotted. It will be noted that at pH less than 2.25 the slope of the two curves differs markedly. Hoffman and Gort-

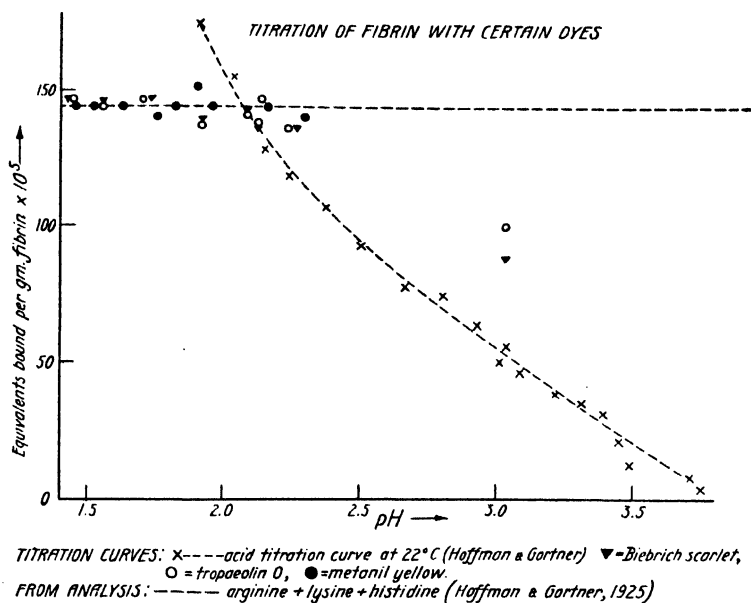


FIG. 7.

ner believe that adsorption of acid by protein takes place in this region. The curves are not strictly comparable since the dye titration curve was carried out at a lower temperature than the Hoffman and Gortner curve. It is not probable that this factor alone is responsible for the difference.

The results of titrating edestin with three different dyes are plotted in Fig. 6. Below pH 2 the titrations average 0.00157 equivalent per gm. of protein which is about 10 per cent more than would be expected from the hexone base content as determined by

Van Slyke (27) in 1911. Since Van Slyke's analysis of the hexone bases in casein and fibrin have since been found to be low it seems quite probable that his values for edestin obtained with the technique in use at that time may also be low. Hitchcock's (33) titration curve assigns a still lower acid-binding value to edestin.

Fibrin was titrated with three dyes. The curves are plotted in Fig. 7. The concentration of fibrin in the solution was 0.038 per cent. The analysis of Hoffman and Gortner was used for the calculation of the theoretical curve. The experimental curve coincides with the theoretical curve. It indicates that fibrin can bind 0.00145 equivalent of acid. Hoffman and Gortner's titration curve is plotted for comparison. The dye titration curve shows no adsorption region analogous to that found by Hoffman and Gortner.

#### DISCUSSION.

(a) *Combining Capacity of Proteins at High Acidity.*—If instead of titrating a protein with a simple acid such as HCl an organic acid such as an acid dye be used, the dye would be expected to react similarly to the simple acid. In the following discussion a comparison of the reaction between simple acids and proteins will be made with the analogous reaction between acid dyes and proteins since it is hoped that this comparison may bring out evidence as to the nature of the combination between dye and proteins. The fact that certain acid dyes form insoluble precipitates with certain proteins is particularly advantageous in studying a problem of this nature. The removal of protein by the formation of an insoluble precipitate with the dye will undoubtedly carry the reaction between dye and protein further to completion than if the end-product were soluble. The fact that each of the proteins titrated to the same equivalence irrespective of the dye which was used in the titration indicates that the reaction takes place in stoichiometric proportions. In no instance was the use of a dye abandoned because it did not fit in quantitatively with the rest of the data; the results represent all of the quantitative data at hand.

Experiments have also been carried out to determine the effect of time and temperature on the amount of dye which can combine with certain proteins in solution. The results are given in Tables



TABLE V.

*Titration of Naphthylamine Brown against a Gelatin Solution.*

75 cc. of naphthylamine brown were added to 0.086 gm. of gelatin.

Time during which mixture stood before filtering.	Temperature.	Dye precipitated by 0.086 gm. gelatin.
hrs.	°C.	cc.
0.5	10	64.3
6	10	63.7
24	10	65.6
5 days	20	64.2

TABLE VI.

*Titration of a Gelatin Solution with Tropaeolin O.*

The tropaeolin O solution was more dilute than the solution used in Table III.

Tropaeolin O added to 0.086 gm. gelatin.	Time during which mixture stood before filtering.	Temperature.	Dye precipitated by 0.086 gm. gelatin.
cc.	hrs.	°C.	cc.
16	0.5	10	10.68
16	24	22	10.68
17	0.5	10	10.93
17	24	22	10.61
18	0.5	10	10.70
18	24	22	10.70

TABLE VII.

*Titration of a Fibrin Solution against Tropaeolin O.*

0.1 N HCl added.	Tropaeolin O precipitated by 1 gm. fibrin.	Remarks.
cc.	equivalents	
4	0.001472	Titrated at 10°C., filtered immediately.
4	0.001478	Let stand 27 hrs. at 10°C. before filtering.
2	0.001475	Titrated at 10°C., filtered immediately.
2	0.001475	Let stand overnight at room temperature (at about 20°C.) and then filtered.

V to VII. They indicate that within the limits of the experimental conditions the amount of dye which precipitates a given amount of gelatin or fibrin is independent of time and temperature.

The combining capacity of the proteins for the acid dyes in our experiments is somewhat greater than the values which have been

reported for the combining capacity of the same proteins for strong acids. It does not appear probable that the mode of union between proteins and these two types of anions should differ. The difference in behavior of protein to dyes and strong acids can be explained on the basis of solubility. The protein acid combinations are soluble while the protein dye compounds are insoluble. The amount of the former which will exist in a solution will depend on the acidity of the solution. If to a similar solution of protein in strong acid dye be added, the dissociated protein ions will be removed from solution as an insoluble precipitate. Due to the acidity of the solution more protein will dissociate and combine with dye. The total amount of dye which the protein will take up at about pH 2 will therefore be greater than its combining capacity for strong acids.

The experiments show that the amount of dye which combines with a particular protein bears a definite relationship to the content of the basic amino acids in the protein molecule. Bracewell (34) and also Greenberg and Schmidt (35) attempted to account for the acid-combining power of proteins on the basis of the arginine and lysine content. The assumption precluded that at pH 1 the basic groups of these amino acids are completely dissociated. If the dissociation curves of lysine, arginine, and histidine be plotted, it will be found that at pH 1 the second basic group of the former are dissociated to about 90 per cent while the second basic group of histidine is about 80 per cent dissociated. The figures are in a sense approximations since at this high acidity no accurate experimental values are available. Using the value found by dye titration as 100 per cent Hitchcock's (26) titration curves of gelatin show that at pH 1 it is dissociated to the extent of about 84 per cent. This value is of the same magnitude as that of the three basic amino acids. While it might be expected that the strengths of the basic amino radicles would be altered by placing the amino acids containing them into a protein molecule it seems improbable that they will be altered in such a manner that all of the radicles would become stronger bases. Michaelis (36) briefly illustrates the situation by stating "wenn man z.B. in das Aniline eine zweite Aminogruppe einführt so wird die Basizität der ersten Aminogruppe verstärkt und die Basizität der zweiten Aminogruppe ist sehr schwach." The algebraic sum of the strength

of the basic amino groups in the protein molecule will probably not differ materially from the algebraic sum of the constants for the second basic groups in the individual amino acids.

The dissociation of the basic groups of the protein molecule will be more complete when dye is added than when a strong acid is added such as HCl. For purposes of calculation we can assume that the dissociation of the basic groups is complete. The protein-dye titration curves show that the amount of dye which is combined in the protein molecule can be correlated with the free basic groups of arginine, lysine, and histidine. Methylation experiments (37) have shown that these groups are probably uncombined in the protein molecule. Cohn (38) has found no objection to the assumption that the acid-combining capacity of the proteins is due to the free basic groups of the hexone bases and the experiments of Felix and Harteneck (39) point in the same direction.

(b) *Combination Near the Isoelectric Point.*—According to the definition of the term isoelectric point given by Michaelis there is no reason to assume that all proteins are totally undissociated at their isoelectric points. He shows that if the amphoteric electrolyte is dissociated at the isoelectric point the slope of the titration curves will be steep at the pH of that point. The acid and base titration curves of gelatin have steep slopes at the isoelectric point. It appears that gelatin may be dissociated both as an acid and as a base at the isoelectric point. Upon the assumption that the dissociation curve of a weak electrolyte is sigmoid Atkin and Douglas (17) went so far as to extrapolate the acid and basic titration curves beyond the isoelectric point for gelatin. It appears that gelatin is appreciably dissociated as a base at pH 5.8 to 6.0. We have found the most basic regions in which a true precipitate between gelatin and dye was obtainable were pH 5.5 for Biebrich scarlet and 4.8 for naphthylamine brown. Grollman (15) found that phenol red united with gelatin at pH 5.4. Loeb (14) also noted that at the isoelectric point and even when the acidity was on the alkaline side of the isoelectric point all of his gelatin granules did not give off their stain. He attributed this to experimental shortcomings. His results can be explained on the basis that gelatin is dissociated at its isoelectric point. Loeb's experiments with gelatin and inorganic ions were probably not

carried out with sufficient refinement to have shown a similar phenomenon.

(c) *Solubility Relations*.—On the assumption that the acid-combining power of proteins is due to the free basic groups of arginine, lysine, and histidine it has been shown that at pH 1 proteins are probably not completely ionized. The theory has been advanced that the dye will completely unite with those basic radicles if the dye protein compound is sufficiently insoluble. It appears desirable, then, to investigate just how insoluble the dye-protein compound must be in order to unite completely with the basic radicles as weak as the weakest of the second basic groups of the hexone bases at a pH of 2 or less, since our data show that the proteins used already combine with a maximum amount of dye at this acidity. Since we do not know the valence of a protein molecule, and since a number of investigators (17–19) have shown that certain proteins acting as bases ionize as though they were mono-acidic bases, it is probable that the equation  $\text{POH} = \text{P}^+ + \text{OH}^-$  may represent the ionization of a protein as a base. In this equation POH represents the protein base, and  $\text{P}^+$  represents the protein ion.

Also,



where  $\text{D}^-$  represents an acid dye ion and PD represents the protein-dye compound.

Then,

$$(\text{P}^+) (\text{D}^-) = K_s$$

is the solubility product of the protein-dye precipitate.

If we treat the protein as if it were a weak base,  $\frac{(\text{P}^+) (\text{OH}^-)}{(\text{POH})} = K_b$ .

Now let  $S$  = total gelatin in solution; therefore,  $S - \text{P}^+ = \text{POH}$ ,

$$(\text{OH}^-) = K_b \frac{(S - \text{P}^+)}{\text{P}^+}$$

$$\frac{(\text{OH}^-)}{K_b} = \frac{S}{\text{P}^+} - 1$$

$$\text{P}^+ = \frac{S}{\left(\frac{\text{OH}^-}{K_b} + 1\right)} \quad (1)$$

But from the solubility product

$$P^+ = \frac{K_s}{D^-} \quad (2)$$

Combining (1) and (2) we get,

$$\begin{aligned} \frac{K_s}{D^-} &= \left( \frac{OH^-}{K_b} + 1 \right) \\ K_s &= \frac{(S) (D^-)}{\left( \frac{OH^-}{K_b} + 1 \right)} \end{aligned} \quad (3)$$

Even though the theoretical reasoning by which this formula is derived may not be strictly correct due to the unknown valency of the proteins, it still must be accepted as an empirical formula which the dye-gelatin compounds follow as will be presently shown. As cited above the treatment of a protein as an ion of low effective valence is not original with this paper, for several investigators have shown that certain proteins behave as though they were monovalent. Cohn and Hendry (18) found that casein from its solubility in NaOH acts as though it were a dibasic acid.

From this formula it is possible to calculate the greatest solubility which PD can have in order to be completely precipitated at a pH of 2 if  $K_b$  is taken as the dissociation constant of the weakest basic group of the hexone bases; namely, that of the second basic group of histidine ( $5 \times 10^{-13}$ ). With gelatin at a pH of 2 an end-point was obtained with Biebrich scarlet in which the dye concentration was estimated as being less than 0.000007 equivalent per liter and the concentration of gelatin was probably less than 0.001 gm. in 100 cc. = 0.00001 equivalent of gelatin per liter. If we assume that the dye is totally dissociated at pH = 2, then 0.000007 equivalent per liter =  $D^-$ . This is probably true since the concentration of dye is low and the dye containing a sulfonic acid radicle is quite a strong acid. By substituting these values in the formula it is possible to estimate what the greatest possible value of  $K_s$  has to be in order for this end-point to be a true one.

$$K_s = \frac{(0.00001)(0.000007)}{\left(\frac{10^{-12}}{5 \times 10^{-12}} + 1\right)} = 2.3 \times 10^{-11}$$

This calculation is based upon a rough estimation and indicates that  $K_s$  must be  $2.3 \times 10^{-11}$  or less, in order that at the end-point obtained (at a pH of 2) the dye will completely precipitate any base whose dissociation constant is  $5 \times 10^{-12}$ . The above is only an approximation because the concentration of dye and gelatin at the end-point is too small to be analyzed in the case of gelatin and Biebrich scarlet or naphthylamine brown. Although the estimate was made for Biebrich scarlet it can be roughly applied to naphthylamine brown because naphthylamine brown also gives a colorless region of about the extent of that of Biebrich scarlet. We have no reason to think that  $K_s$  for gelatin-Biebrich scarlet is the same as that of gelatin-naphthylamine brown; nevertheless, the values in case of both dyes should be less than the estimated value (*i.e.* of  $K_s = 2 \times 10^{-11}$ ).

A check on this estimation was made by actually determining the concentration of gelatin and dye at a pH at which the gelatin-dye compound is sufficiently soluble to exist in sufficient quantities to be analyzed quantitatively. It is obvious that the protective colloidal region cannot be used for this purpose for it does not give the solubility of the dye-gelatin precipitate but rather the solubility of some soluble form of dye-gelatin compound, or dye-gelatin complex. (An excess protein might exert a slight protective effect even under the conditions employed; however, such a phenomenon would cause the real solubility product to be still smaller than the apparent one, so that the error in such a case would be in the direction which favors this argument.) The above requirements were met by making the determinations at a pH near the isoelectric point and by using sufficient dye to avoid the protective colloidal region. Naphthylamine brown and Biebrich scarlet were selected since they give colorless regions. Consequently, the protective colloidal effect does not exist over a considerable region. This fact suggests that the protective colloidal effect may be a minimum when these dyes are used.

Three determinations were carried out with Biebrich scarlet and two determinations with naphthylamine brown and gelatin at a

pH near the isoelectric point. The results are given in Table VIII. The value for the dissociation constant of gelatin is taken as that of the second  $K_b$  of histidine, which is  $5 \times 10^{-13}$ . In all calculations the equivalence of gelatin was considered as 0.001 per gm. as an approximation, since the acid titration curves of various investigators and the dye titration curves give approximately that value as the acid-combining capacity of gelatin. It will be observed that the two determinations with gelatin-naphthylamine brown are of the same order of magnitude even though there is 111 per cent variation from the mean. The greatest variation from the mean in the three determinations with Biebrich scarlet is about 14 per cent.

TABLE VIII.  
*Solubility Products of Gelatin-Dye Compounds.*

Dye.	Concentration of gelatin $\times 10^6$ .	Concentration of dye $\times 10^6$ .	Concentration ( $\text{OH}^-$ ) $\times 10^{11}$ .	pH	$K_s \times 10^{12}$ .
	<i>equivalents per liter</i>	<i>equivalents per liter</i>			
Naphthylamine brown.	33	78	69.3	4.84	1.85
“ “	26	50	10.7	4.02	5.95
Biebrich scarlet.	21	33	3.88	3.58	9.10
“ “	77	105	41.6	4.62	9.90
“ “	130	380	329	5.51	7.65

It will be noted that not only are the solubility products in all cases less than the value which our rough estimates require them to be, but for each dye they agree as well as the great error involved in determining low concentrations of dye and gelatin will permit. If gelatin may be considered to act as a monoacidic base it appears that the solubility products of gelatin-naphthylamine brown and gelatin-Biebrich scarlet are sufficiently low to permit a quantitative union with the weakest second basic group of the hexone bases of gelatin at pH 2.

The arguments which have been advanced tend to support the idea that the union between the proteins and dyes which were studied is chemical in nature. The dye-combining capacity of the proteins has been carried to acidity regions corresponding to pH 1. The dye titration curves of gelatin, casein, and fibrin differ from

the acid titration curves which have been obtained by Hoffman and Gortner (32). These authors conclude that between the acidities of pH 2.5 and 10.5 the combination between protein and acid is chemical while outside of these limits it is an adsorption type. Hoffman and Gortner have pointed out that in regions of extreme acidity and alkalinity the error in the estimation of acid- and base-binding power is considerable. To this must be added the factor of uncertainty in the value of the contact potential. This becomes more pronounced in regions of high acidity and alkalinity. Cohn (40) has recalculated certain of Hoffman and Gortner's measurements using the activity coefficients of Lewis and Randall. He concludes that the assumption of adsorption is unnecessary. Cohn's recalculations however neglect the influence of the protein on the activity of the hydrogen ion. No data are at present on hand on which to base such a calculation. The use of dyes to determine the combining capacity of proteins is not subject to the same criticism as when strong acids and bases are used for this purpose. The electrometric estimations are not vital to the measurements. They are used for reference purposes only and errors in the estimation of acidity do not affect the conclusions. The results do not warrant an assumption that the combination between the proteins and dyes which were used in these experiments is, within the limits of the experimental conditions, an adsorption phenomenon, or that the -COHN- groups play a rôle (41). The data can be explained on the basis of a stoichiometric reaction between the proteins and dyes.

#### SUMMARY.

1. Experiments were carried out to determine the behavior of certain proteins with a number of acid dyes.
2. Gelatin granules were found to be unsatisfactory for quantitative estimation of the amount of dye with which this protein can combine. Solutions of proteins were found satisfactory for this purpose.
3. The data indicate that the combination between the proteins and dyes used in these experiments takes place in stoichiometric proportions. The combining equivalence of proteins for dyes is slightly greater than for acids. Gelatin was found to combine with



certain dyes on the alkaline side of the isoelectric point. Reasons have been advanced to explain this phenomenon.

4. The capacity of the proteins to combine with dyes can be correlated with the content of the free basic groups of arginine, lysine, and histidine.

5. The data do not support the assumption that in acidities lying between pH 2.5 and 1.0 the combination between protein and dye is an adsorption phenomenon or that the -COHN- groups play a rôle.

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## NOTE ON THE EFFECT OF POTASSIUM IODIDE IN THE SHAFFER-HARTMANN MICRO SUGAR REAGENT.

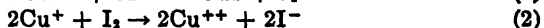
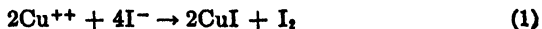
By W. A. DeLONG.

(From the Chemical Laboratory of Macdonald College, McGill University,  
Montreal, Canada.)

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It has been observed by Duggan and Scott<sup>1</sup> that in combining the copper-carbonate and iodide-oxalate fractions of the Shaffer-Hartmann micro sugar reagent there results a decrease in sensitivity. This decrease is particularly evident, of course, in solutions of low concentration in reducing sugars, such as are met with in the estimation of blood sugar. That the copper reduction of this reagent is so diminished was brought to the writer's attention through the use of the Shaffer-Hartmann method in checking the concentration of sugar standards for use in colorimetric work. Duggan and Scott, while noting the lessened delicacy and suggesting that the cause was probably the addition of either the potassium oxalate or the potassium iodide, did not attempt to determine which of these added substances was responsible. The work outlined below was undertaken with this end in view.

The principal reactions involved in the determination of copper reduction by sugar solutions under the conditions prescribed by Shaffer and Hartmann<sup>2</sup> are, according to these authors, as follows:



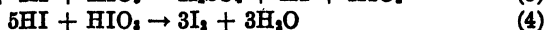
They state further that reaction (1) is inhibited more or less completely by the addition of alkali oxalates, alkali cuprioxalates being formed, which dissociate to give alkali cations and cuprioxa-

<sup>1</sup> Duggan, W. F., and Scott, E. L., *J. Biol. Chem.*, 1926, lxxvii, 304.

<sup>2</sup> Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, xlv, 349, 365.

## 732 Shaffer-Hartmann Micro Sugar Reagent

late anions. The addition of alkali oxalates therefore reverses reaction (1) completely in the presence of free iodine. The latter is produced according to the equations:



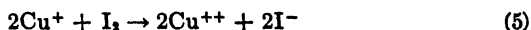
Therefore, when after partial reduction of an alkaline copper solution by reducing sugars in the presence of potassium oxalate, iodide, and iodate, the solution is acidified with sulfuric acid, the HI liberated reduces the iodic acid to free iodine, but has no

TABLE I.

*Effect of Order of Addition of Ingredients of the Shaffer-Hartmann Micro Sugar Reagent upon the Quantity of Copper Reduced.*

Experiment No.		Thiosulfate equivalent of Cu reduced.	Per cent decrease or increase.
		cc. 0.008N	
1	Combined micro reagent.....	5.31	
2	Iodide, iodate, and oxalate added <i>after</i> reduction.....	5.52	+3.95
3	Oxalate added <i>after</i> reduction.....	5.20	-2.07
4	Iodide and iodate added <i>after</i> reduction.....	5.85	+10.17
5	Iodate added <i>after</i> reduction.....	5.13	-3.39
6	Iodide " " ".....	5.54	+4.33
7	Iodate and oxalate added <i>after</i> reduction.....	5.10	-3.95
8	Oxalate " iodide " " ".....	5.63	+6.03

effect upon the unreduced copper. The *cuprous copper*, however, is oxidized by the free iodine, thus:



The excess of iodine in the solution is then estimated by the use of standard thiosulfate solution. From this value and the amount of iodine liberated from the iodate in a blank determination, the iodine consumed in the reoxidation of the copper, and consequently, the amount of copper reduced by the sugar, may be calculated.

Observations on the respective effects of the iodide and oxalate added in the combined reagent were made in the following manner.

1 cc. of 0.1 per cent glucose solution and 4 cc. of the combined reagent, or a modified form of the latter, were heated in loosely stoppered test-tubes in a boiling water bath for 15 minutes, cooled under the tap for 3 minutes, and 5 cc. N sulfuric acid added. After 1 minute, the liberated iodine was titrated with 0.005 N thiosulfate solution. The number of cc. of 0.005 N thiosulfate equivalent to the copper reduced in each case is given in Table I.

It would appear from the results obtained that the addition of the potassium iodide was responsible for the diminution in sensitivity to reducing sugars shown by the Shaffer-Hartmann *combined* copper reagent. This is indicated by Experiments 2, 4, 6, and 8, in all of which the iodide was added *after* reduction, since

TABLE II.

*Advantages of Adding the Potassium Iodide after Reduction, Particularly where the Concentration of Reducing Sugars Is Small.*

	Thiosulfate equivalent (cc. 0.005N) of copper reduced by 1 cc. glucose solution of:		
	0.05 per cent.	0.025 per cent.	0.010 per cent.
KI added <i>before</i> reduction.....	2.35	1.03	0.35
" " <i>after</i> " .....	2.48	1.23	0.43
Approximate increase, <i>per cent.</i> .....	5	20	25

each of these shows an *increase* in the copper reduced by 1 cc. of 0.1 per cent glucose solution over that obtained in Experiments 1, 3, 5, and 7, in which the iodide was added *before* reduction took place.

The sensitivity of the reagents to sugar solutions containing approximately 50, 25, and 10 mg. of glucose per 100 cc. respectively was also tested when potassium iodide was present *during* the reduction process, and when it was added *after* reduction was complete. The results are shown in Table II.

A further consideration of Table I might seem to indicate, however, that the potassium oxalate plays a rôle in the increase of reduction. For example, in Experiment 4, in which the oxalate is added *before* reduction and the iodide *after*, the amount of copper reduced is considerably greater than is the case with any

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other combination. This is substantiated in a negative way by Experiments 3 and 7, in which the oxalate is added *after* reduction, and in which the thiosulfate equivalent of the copper reduced is *correspondingly low*. Experiment 8, on the other hand, indicates that the inhibitive effect of the iodide is more responsible for the low values obtained in Experiments 3 and 7 than is the addition of oxalate *after* rather than *before* reduction.

A rapid falling off in delicacy with decrease in concentration of glucose when the potassium iodide is added *before* reduction takes place is thus clearly indicated.

It is concluded from the foregoing results that the addition of potassium iodide prior to reduction is the chief factor operating to decrease the sensitivity of this reagent. The writer has no adequate explanation of this phenomenon to offer. As Shaffer and Hartmann<sup>2</sup> have shown, the effect of presence of the iodide is to *increase* the proportion of cupric salt reduced to cuprous iodide, according to the mass law equation

$$(\text{Cu}^{++}) \cdot (\text{I}^-)^2 = \sqrt{I_2} \cdot K$$

Moreover, no free iodine could be detected in the combined reagent as a result of the addition of iodide to the mixture. However, although the mechanism of the reduction of sensitivity remains obscure, the defect may be overcome, as has been shown above, by the addition of the potassium iodide *after* reduction has been effected. The micro reagent may be made up as usual, omitting the iodide, which is later added in a solution of appropriate concentration.

### SUMMARY.

The effect upon the sensitiveness of the Shaffer-Hartmann micro carbonate-tartaric acid reagent of the addition of potassium iodide, iodate, and oxalate has been studied.

It is concluded that the addition of potassium iodide is the chief factor operating to decrease the delicacy of this reagent.

It is shown that a reagent much more sensitive in the lower ranges of glucose concentration may be obtained by omitting the potassium iodide from the combined reagent and adding this material after reduction of the copper reagent has taken place.

Standardizations of the modified reagent against glucose solutions of known concentration is, of course, essential, as Duggan and Scott have emphasized, in order to obtain correct values for glucose under the changed conditions.

This work was done during the tenure of an assistantship under Dr. J. F. Snell, Macdonald College, McGill University, to whom the author wishes to express gratitude for the facilities offered.





# A MODIFIED ELECTRO-GUTZEIT APPARATUS FOR THE QUANTITATIVE ESTIMATION OF MINUTE AMOUNTS OF ARSENIC IN INSECT TISSUE.

By DAVID E. FINK.

*(From the Truck Crop Insect Investigations, Bureau of Entomology, United States Department of Agriculture, Washington.)*

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In studying the effect of arsenicals upon insects it is essential to estimate the amount of arsenic absorbed by the protoplasm of tissues and organs. Inasmuch as the quantity absorbed is of a magnitude not discernible by ordinary titration methods, the writer found it necessary to use the more sensitive Gutzeit method. Several objections appeared when this method was used, the most outstanding one being the fact that blank runs usually registered traces of arsenic, which was due to the As in the zinc essential in the generation of hydrogen. To eliminate this difficulty it was necessary to adopt the electrolytic method of generating hydrogen by the electrolysis of sulfuric acid. This necessitated the use of an electrolytic apparatus, of which there are several described in the literature (Thorpe (1), Trotman (2), Sand and Hackford (3), Mai and Hurt (4), Monier-Williams (5), Lawson and Scott (6)), all of them based on the principle that hydrogen when evolved electrolytically in the presence of arsenious acid or its salts forms arsine ( $\text{AsH}_3$ ).

When the writer first investigated the subject, an apparatus was designed in many respects similar to the modified Gutzeit apparatus described by Lawson and Scott (6), which consisted of a porous cell, the cathode chamber, set in an outer glass vessel, the anode chamber. The same strength of electrolyte ( $\text{H}_2\text{SO}_4$ ) was used in both chambers; the cathode chamber contained, in addition, the solution to be investigated. The latter was connected with an outlet tube to serve as a holder for the mercuric bromide strips receiving the arsine stain. Blank tests conducted

with such an apparatus gave no stain of arsine on the mercuric bromide strips, indicating that the reagents were free from this element.

But it was soon discovered that in the evolution of arsine from solutions containing equal quantities of arsenic, variations in the stains of the paper strips took place. Obviously some of the arsenic was retained by the apparatus. Considerable effort was made to locate the source producing the variations, and finally suspicion centered upon the absorption and retention of arsenic by the porous cup. To determine the coefficient of absorption of the porous cup did not appear feasible, since different cups might be expected to have different absorption coefficients. To eliminate the porous cup altogether, seemed a better procedure. This was done by modifying the apparatus so that a glass cylinder fitted into a slightly larger glass vessel, the former to serve as cathode, the latter as anode chamber. Trotman (2) tied a parchment membrane around the bottom of the cathode cylinder for the purpose of separating the solution in the cathode from that in the anode chamber. But this is not essential, since the hydrogen generated can pass out as arsine only from the cathode chamber.

Further modification was but a logical step toward compactness and simplicity and as a result the apparatus resembles the one described by Mai and Hurt (4), but differs from it in many respects since it is especially designed for the estimation of minute amounts of arsenic likely to be encountered in tissue and organs of insects. This electrolytic apparatus may also be found of considerable value in other fields of investigation; it ought to be of importance especially to the physician in determining arsenic in urine and blood.

#### *Description.*

In structure the apparatus resembles a U-tube, the arms serving as cathode and anode chambers, of the following dimensions: height 11 cm., inside diameter 1 cm., volumetric capacity 15 cc. It is made of Pyrex glass fitted with hollow ground glass stoppers in which platinum wires are sealed for connection with cathode and anode electrodes, the former a strip of pure sheet lead  $1 \times 2.5$  inches, the latter a strip of platinum foil of the same dimen-

sions. Both electrodes are rolled to fit the inner diameter of the apparatus and are held in place by copper clips soldered to copper wires, which in turn are welded to the platinum wires sealed in the glass stoppers (Fig. 1). Fused to the outlet from the cathode side is a tube 5 mm. in diameter and about 4 cm.

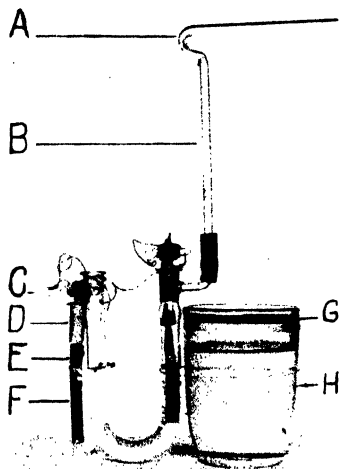


FIG. 1. The U-shaped apparatus and tumbler used as water bath. *A*, the holder for the strips of filter paper receiving the arsine stain; *B*, the portion of tube containing loosely plugged cotton previously soaked in lead acetate and dried; *C*, the outlet from the anode chamber; *D*, the ground-glass stopper through which a platinum wire leads to connection with the copper clip; *E*, the copper clip soldered to the wire; *F*, a strip of rolled platinum foil held by the clip which serves as an anode electrode; *G*, the Bakelite for holding the apparatus when immersed in the water bath of the tumbler; *H*, the tumbler used as a water bath.

long, which is bent at right angles to the U-tube and communicates with the interior by means of an opening in the glass stopper. This side tube connects also with a 4 mm. glass tube about 24 cm. in length, the lower portion of which is stoppered loosely with cotton previously soaked in a 20 per cent solution of lead acetate and dried, and the upper end is bent to serve as a holder for the

filter paper strips. Fused to the anode chamber is a tube 4 cm. in length which serves for the escape of the oxygen. The apparatus is placed in a tumbler which serves as a water bath and by means of Bakelite is maintained in an upright position. The tumbler containing the apparatus in turn is set in a larger water bath. The double water bath serves to reduce the rise in temperature occasioned in the generating of the arsine. Such a unit as described above is termed a cell and is connected in series with a set of resistance lamps, the latter connected in parallel. As many cell units as desired may be placed in series to enable several determinations to be made at one time. The writer found it convenient to use three cells.

#### *Procedure.*

To determine the strength of electrolyte to use in the generating of hydrogen, experiments were conducted with various dilutions of sulfuric acid (95 per cent, sp. gr. 1.83), and it was noted that a 12.5 per cent dilution gave the best results. The usual procedure in obtaining standards was to use 10 cc. of sulfuric acid to which 1 to 2 cc. of the solution to be tested for arsenic were added, placed in the apparatus, and electrolyzed with a direct current of 1 ampere and 11 volts. If the solution under investigation contained arsenious acid or sodium arsenite in concentrations of 0.01 mg. or less, a half hour was usually sufficient for complete evolution of the arsine, and a correspondingly greater length of time for higher concentrations. If the solution to be tested contained arsenic acid or sodium arsenate, the required time for complete evolution of the arsine was considerably greater.

To test for arsenic in organic matter, as in muscle tissue or organs of an insect, the writer found it convenient simply to grind the organic matter in several cc. of the electrolyte used for the generating of the hydrogen, and afterwards the volume of the solution was made up to 10 cc. by the addition of further amounts of the sulfuric acid and placed directly in the apparatus and electrolyzed. In experiments involving the determination of arsenic in organic matter it is essential to add 1 to 2 cc. of amyl alcohol in the apparatus to prevent foaming of the solution during electrolysis.

To make certain that the usual procedure recommended for destroying organic matter was not necessary when dealing with

insect material before electrolytically testing for arsenic, the following experiments were made: Samples of insect tissue containing known amounts of arsenic were first reduced in the ordinary way as described by Lawson and Scott (6), before placing in the apparatus to be electrolyzed, and other experiments made with insect tissue containing the same amount of arsenic were not previously reduced, but simply ground up in the manner discussed above and placed directly in the apparatus. The arsine stains recorded from these two tests were compared and the latter method, without destruction of organic matter, was found to give a stain superior in uniformity, intensity, and area.

If it is desired to test for arsenic in materials of much larger bulk than is found in the tissues and organs of single insects, as, for example, leaves, or fruit of plants, it is essential to increase the capacity of the apparatus to 50 or 75 cc. The electrodes should also be increased in surface area.

The electrodes after each determination are made sensitive by immersing in dilute warmed nitric acid for several minutes, and afterwards washed thoroughly in water. The apparatus similarly is readily cleaned with a test-tube brush and by rinsing several times with water.

The apparatus is sensitive to 0.00002 mg. of arsenious acid, and 0.001 mg. of arsenic acid.

#### *Selection of Paper Strips.*

Care in the selection and preparation of the filter paper strips is of importance to assure uniform stains. A large series of different types of filter and non-filter paper was tried out experimentally, and a Whatman No. 5230 filter paper of a hard uniform texture, which comes in sheets, gave the best results. This paper is first cut into strips 4 mm. in width and of the desired length by means of a good photographic trimmer and afterwards soaked in a 1.5 per cent mercuric bromide solution dissolved in absolute alcohol. To obtain uniform stains it is essential to place the cut strips in the solution mentioned above for several hours and to dry them for 10 minutes in a desiccator, or for several minutes by waving in the atmosphere just previous to each test. If the strips become too dry the stain will not record uniformly.

In placing the strips in the holders of the apparatus previous to a test, the end receiving the stain should be exactly centered to insure a uniform stain on both sides of the strip. After each test, if the strips are dipped in a 10 per cent solution of potassium iodide, a sharp end-point is obtained.

Standards should be prepared from known amounts of arsenic in insect tissue, so that unknowns obtained from the same source may be comparable to the standards. This is readily accomplished, as it is a simple procedure to grind the organic matter with known amounts of arsenic to be used as standards.

For the quantitative estimation of known amounts of arsenic in a sample, advantage is taken of the fact that the upper limits of the stains for correspondingly increasing amounts of arsine lie upon a straight line. By carefully calculating the area on a large series of standard strips obtained from known amounts of arsine and determining carefully the slope of the line, strips containing unknown amounts of arsenic may be superimposed upon the standards or their amount determined from the slope of the line.

#### SUMMARY.

An electrolytic apparatus which has been designed chiefly for the determination of minute quantities of arsenic in insect tissue is described. Tests for trivalent or pentavalent arsenic in insect tissue are made directly with this apparatus without previous reduction or destruction of organic matter. Quantitative estimations of unknowns are determined from the slope of a line produced from a large series of standards obtained from known quantities of arsenic in insect tissue. The apparatus is sensitive to 0.00002 mg. of arsenious acid, and 0.001 mg. of arsenic acid. By increasing the capacity of this apparatus from 15 to 50 cc., tests for residual arsenic on sprayed foliage or fruit may be made.

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## OXYADENINE.

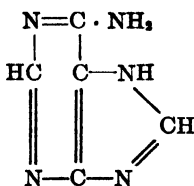
BY MARY V. BUELL AND MARIE E. PERKINS.

(From the Laboratory of Physiological Chemistry, the Johns Hopkins University, Baltimore.)

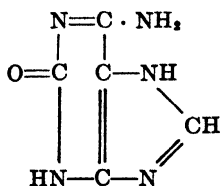
(Received for publication, February 4, 1927.)

### INTRODUCTION.

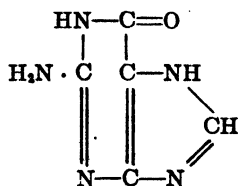
In 1897 Fischer (1) synthesized a large number of purines, some of which (adenine, guanine, *et al.*) were known at that time to occur in nature. He pointed out the striking similarity between 2-amino-6-oxypurine (guanine) and its synthetic isomer 2-oxy-6-amino purine, and also stated that from its constitution, 2-oxy-



6-Aminopurine  
adenine.



2-Oxy-6-aminopurine  
oxyadenine.



2-Amino-6-oxypurine  
guanine.

6-aminopurine must be regarded as an oxidation product of adenine. Because of this relation to adenine Fischer predicted that 2-oxy-6-aminopurine would be found in the animal organism and suggested that it might already have been mistaken for guanine.

That Fischer's prediction has been fulfilled will be shown in this paper. 2-Oxy-6-aminopurine has been isolated from pig blood and has been called *oxyadenine*.

### EXPERIMENTAL.

*Isolation of Oxyadenine Chloride from Pig Blood.*—In principle the preparation of oxyadenine chloride from blood is simple and establishes, beyond a doubt, the purine nature of the compound.

From the protein-free blood filtrate the combined purines (nucleotides, etc.) were precipitated as uranyl compounds, which were hydrolyzed with sulfuric acid. The free purines were precipitated first as cuprous compounds and then as silver compounds in the presence of ammonia. After the silver was removed with hydrochloric acid, the solution was evaporated until oxyadenine chloride was precipitated.

*Detailed Method.*—Fresh defibrinated pig blood was added to 5 volumes of boiling 0.01 N acetic acid containing small amounts (2 gm. per liter) of crystalline sodium acetate. The solution was boiled for 1 minute, filtered, and to the hot, clear, straw-colored filtrate an excess of uranyl nitrate solution was added (10 cc. of a 20 per cent solution to each liter of filtrate). This procedure was finished within 2 hours after the blood was shed. The yellow precipitate was collected, suspended in hot water, treated with sufficient sulfuric acid to dissolve it, and the solution was boiled for an hour. It was then diluted with about 10 volumes of water, and sodium hydroxide was added until the reaction was only faintly acid toward litmus. The precipitate which formed was removed and washed thoroughly with hot water.

The purines were precipitated as cuprous compounds from this solution by the well known copper sulfate-sodium bisulfite process. The first precipitate of cuprous purine was dark and did not have the characteristic texture of a copper purine compound. It was decomposed in the usual way with sodium sulfide, acidified with sulfuric acid, freed from hydrogen sulfide by boiling, diluted with several volumes of boiling water, and treated again alternately with copper sulfate and sodium bisulfite. This procedure was repeated twice, making a total of four precipitations of the purines as copper compounds. The final precipitate of copper purine behaved as one would expect if one were precipitating the purine from a solution of a pure compound.

The solution was made alkaline to litmus with ammonia, and was treated with ammoniacal silver nitrate solution as long as a precipitate continued to form. The silver purine was filtered and washed with warm water until the presence of ammonia in the wash water could no longer be detected with litmus paper. It was then suspended in hot water and was treated with dilute

hydrochloric acid until it was determined by the character of the precipitate that the silver purine had been completely decomposed. Care was taken not to add an excess of hydrochloric acid. The silver chloride was filtered off and the solution was evaporated on the water bath until a precipitate started to form. It was then allowed to stand overnight at room temperature, exposed to the air. The following day the precipitate, which consisted of hard macroscopic balls of microscopic needles, was filtered off and dried. 10 liters of blood yielded about 50 mg. of these crude crystals of oxyadenine chloride. On further evaporation the mother liquor failed to yield more precipitate until the solution approached dryness. The dry residue consisted principally, if not entirely, of adenine chloride.

The oxyadenine chloride was washed free from adhering traces of adenine chloride by grinding it in relatively large volumes of hot water, faintly acidified with hydrochloric acid. The solution was concentrated to small volume on the water bath, filtered, and the washing process was repeated twice. The final wash water failed to show the presence of even a trace of adenine. The oxyadenine chloride was dissolved in hot 10 per cent hydrochloric acid, and the solution was treated while hot with decolorizing charcoal. From the filtered solution crystallization was allowed to take place. These crystals were beautiful long tapering needles which, when dry, had a glistening fluffy appearance.

*Analysis of Oxyadenine Chloride.*—The following analyses were made by the Pregl micro methods with the help of Dr. O. Wintersteiner, to whom grateful acknowledgment is made.

	Calculated for $C_8H_8N_5O \cdot HCl \cdot 2H_2O$ per cent	Found. per cent
C.....	26.84	27.05
H.....	4.50	4.52
N.....	31.32	31.88
Cl.....	15.86	15.54
H <sub>2</sub> O.....	16.11	16.18

*Free Base.*—A dilute solution of the chloride in hydrochloric acid was made ammoniacal. Free oxyadenine was precipitated immediately, as a semicrystalline powder, which was washed with water, dried, and analyzed.

## Oxyadenine

*Oxyadenine (Free Base).*

	Calculated for $C_5H_5N_5O$ per cent	Found. per cent
N.....	46.36	46.47

*Sulfate.*—Oxyadenine was dissolved in hot dilute sulfuric acid. On cooling the sulfate crystallized as obliquely cut prisms, and did not lose its water of crystallization at 120°.

*Oxyadenine Sulfate.*

	Calculated for $(C_5H_5N_5O)_2H_2SO_4 \cdot H_2O$ per cent	Found. per cent
S.....	7.66	7.69

*Picrate.*—Picric acid was added to a dilute solution of oxyadenine in sulfuric acid. Oxyadenine picrate was precipitated as orange-yellow needles which could be recrystallized from hot dilute picric acid but not from water.

*Murexide Reaction.*—The murexide reaction given by oxyadenine was indistinguishable from that given by guanine.

## DISCUSSION.

Quantitative analyses of the chloride led sharply to the formula  $C_5H_5N_5O \cdot HCl \cdot 2H_2O$ , which is also the formula for guanine chloride. Guanine is known to be 2-amino-6-oxypurine. In spite of many striking similarities between the two compounds, it can be shown that the compound under investigation is not guanine chloride. Guanine chloride loses its water of crystallization quantitatively in a vacuum desiccator at room temperature. Oxyadenine chloride lost no weight when it was heated for several hours at 110° *in vacuo*. It lost its water of crystallization sharply, however, when the temperature was raised to 120°. Prolonged heating at this temperature did not cause further loss of weight. Oxyadenine is more insoluble than guanine and is a weaker base than guanine. Its salts are easily hydrolyzed by pure hot water. A dilute aqueous solution of oxyadenine picrate deposits large diamond-shaped crystals of the free base.

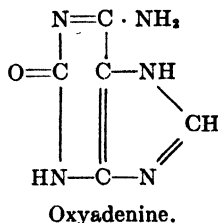
There is the theoretical possibility that the amino group may be in position (8) and oxygen in position (2) or (6). Since these compounds are not known and since the properties of oxyadenine

correspond satisfactorily with those of 6-amino-2-oxypurine, this possibility may be dismissed.

That the amino group should occupy position (6) is to be expected from *a priori* considerations. The principal purine in pig blood is adenine. This fact makes it seem probable that a simple oxidation product of adenine also occurs in this blood. To be sure, the oxygen might occupy position (8) instead of position (2). Fischer (2, 3) prepared this compound also. On analysis of the sulfates of the closely related compounds 6-amino-8-oxypurine and 6-amino-2-oxypurine, he found that the first contained no water of crystallization and the second, 1 molecule of water of crystallization which was not given off at 120°. Guanine sulfate has 2 molecules of water of crystallization which are given off below 120°. Variations in the amounts of water of crystallization produce small but detectable differences in the percentage of other elements in the sulfate, *i.e.* sulfur. The properties of the sulfate of oxyadenine are identical with those described by Fischer for the sulfate of 6-amino-2-oxypurine, as to the sulfur content, stability toward heat (120°), and crystal form.

#### CONCLUSIONS.

A purine has been isolated from pig blood which is identical with Fischer's synthetic 6-amino-2-oxypurine. In spite of its striking similarity to guanine, this compound, which we have called oxyadenine, is not guanine, and must have the following structural formula.



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## ANTIRICKETIC SUBSTANCES.

### VI. THE DISTRIBUTION OF VITAMIN D, WITH SOME NOTES ON ITS POSSIBLE ORIGIN.

By CHARLES E. BILLS.

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.)

(Received for publication, February 5, 1927.)

Knowledge of the distribution of the vitamins is confined largely to vitamins A, B, and C. Concerning the antiricketic factor, D, there is little information, save that it occurs in some fish oils, in egg yolk, and to a small extent in butter fat. The present report summarizes an extensive study of its distribution in various oils and fats, the assays on which are compared in Table I.

The tests were made by the procedure adopted for the commercial assay of cod liver oil; *i.e.*, the minimum dose of oil was determined which sufficed to produce distinct healing in rickety rats during a 5 day period. The oils were administered by trituration with McCollum's Diet 3143, so that the antiricketic intake always corresponded with the variable intake of calcium in the food. Records of over 3000 rats enter into the tabulation, several hundred of which were used for tests on oils other than cod liver oil. Individual protocols need not be given, as the tests were performed under highly uniform conditions.

From the line test findings the relative potencies of the several oils were computed on a scale of 100, taking as the standard the average activity of Newfoundland medicinal cod liver oil. The standard induced distinct (two plus) healing at a level of  $\frac{1}{4}$  per cent. Oils which failed to induce any healing at 4.0 per cent are reported as nil.

Table I reveals that of all the oils examined, only the fish oils and the oil of the adult seal were active. There is a great difference in activity between the oils of different species of fish, even be-



tween related species, and the oil of a given species may vary considerably. The vitamin is not confined to liver oils, for it appears

TABLE I.  
Showing the Vitamin D Potency of Various Oils in Comparison with Cod Liver Oil.

Source and description of oil.	No. of samples.	Relative potency.
Puffer fish liver, supplied and previously described by Hess and Weinstock (9).....	2	1500
Cod liver, Newfoundland medicinal .....	500	100
Goosefish liver ( <i>Lophius piscatorius</i> ), Boston.....	1	100
Herring, Newfoundland.....	4	100
Sardine, California.....	4	100
Menhaden, Chesapeake Bay.....	5	75
Shark liver, Newfoundland.....	1	75
Muddy catfish ( <i>Leptops olivaris</i> ), fatty tissues, Ohio river..	1	40
Coalfish liver (American pollack), Boston.....	9	40
Salmon trimmings, Pacific coast.....	3	20
Haddock liver, Boston .....	5	10
Channel catfish ( <i>Ictalurus punctatus</i> ), visceral fat, Ohio river.	1	6
Squid ( <i>Ommastrephes illecebrosus</i> ), Newfoundland.....	2	6
Caplin ( <i>Mallotus villosus</i> ), Newfoundland.....	4	3
Dogfish liver, Newfoundland.....	2	3
Seal blubber ( <i>Phoca grænlandica</i> ), adult female, Newfoundland.	1	3
Seal blubber ( <i>Phoca grænlandica</i> ), new born, Newfoundland.	3	Nil.
Whale blubber, antarctic, Graham Land..	1	"
Cod liver, commercial hydrogenated.....	2	"
Veal fat, extracted with ether.....	1	"
Oleo, commercial.....	1	"
Coconut, edible white.....	1	"
Cottonseed, edible.....	1	"
Linseed, U.S.P.....	1	"
Maize, edible (from white maize).....	1	"
Olive, " .....	1	"
Peanut ( <i>Arachis</i> ), edible.....	1	"

in the body oil of the herring, menhaden, salmon, etc., in the fat tissue oil of the catfish, and in the blubber oil of the adult seal.

In a previous communication (1) I reported that the blubber oil of the new born seal (*Phoca grænlandica*) is not antiricketic. Last April the manufacturer of the seal oil kindly rendered some oil from a mother seal, and this specimen was found to be slightly antiricketic. Two significant facts are therefore evident: First, the adult seal, living on a strongly antiricketic diet of fish, does not accumulate in its oil a quantity of vitamin D at all commensurate with the quantity in fish oil. Second, either the mother seal does not transfer her store of vitamin D in detectable amounts to her young *in utero*, or else, improbably, the young destroy the vitamin received.

It is the general belief that vitamins originate principally in the vegetable kingdom, for it is in plant tissues that they are most often abundant. Vitamins stored in animal tissues may in many cases be traced to a vegetable origin. Thus the exceptional content of vitamins in fish oil is attributed (2-4) to their abstraction by the fish from marine foods, ultimately vegetable. This explanation of the genesis of vitamin D would be acceptable, were it not for certain contradictory data. To be sure, the vitamin D of egg and butter fats may be attributed to transference from isolated vegetable substances. But as a rule vegetable fats are not antiricketic. The occasional anomalous activity of coconut oil is explained by Steenbock and Black (4) on the ground that this oil is sometimes prepared from sun-dried copra.<sup>1</sup>

Unfortunately information is lacking on the photosynthesis of antiricketic substances in plankton,<sup>2</sup> but it is not difficult to study the distribution of vitamin D in the immediate food of certain fish, such as the cod. In some parts of the world the feeding habits

<sup>1</sup> As early as 1854 Thompson (5), writing on the anemia of tuberculosis, stated that "the use of almond-oil and of olive-oil was not followed by any remedial effect, but from the cocoa-nut oil results were obtained almost as decided as from the oil of the liver of the Cod . . . ." His preparation was undoubtedly made from sun-dried copra, for it was "a pure cocoa oleine, obtained by pressure from crude cocoa-nut oil, as expressed in Ceylon and the Malabar coast from the *Copperah* or dried cocoa-nut kernel, and refined by being treated with an alkali and then repeatedly washed with distilled water."

<sup>2</sup> The work of Jameson, Drummond, and Coward (6) and Coward (7) indicates that diatoms and algæ are capable of synthesizing a growth-promoting factor, probably vitamin A.

of the codfish are especially well defined, and open to analysis. For instance, during the period of the most rapid elaboration of oil in the Newfoundland cod,<sup>8</sup> the fish feed almost exclusively on the caplin (*Mallotus villosus*) which abound in the coastal waters during midsummer.

From studies made at the fisheries, and from a mass of data obtained in the manufacture of cod liver oil, I have computed that the liver of the average (3.5 kilos) codfish gains roughly 57 gm. or 116 per cent of ether-extractable matter during the 4 weeks following June 25. While the amount of oil thus increases, its potency is not diminished. In fact, assays on many commercial samples of this oil tend to show that the amount of vitamin D per gm. of oil is slightly augmented.

Now the caplin is a conspicuously poor source of vitamin D. Four samples of caplin oil prepared from the entire fresh fish were found to be only  $\frac{1}{3\frac{1}{2}}$  as active as the average cod liver oil. That is, 4.0 per cent of caplin oil was the antiricketic equivalent of  $\frac{1}{3}$  per cent of cod liver oil. Moreover, the caplin contains very little oil. Analyses kindly furnished by Mr. D. James Davies of the Newfoundland Government Laboratory show that the entire fish contains less than 2 per cent of ether-extractable matter.

From these data it may be calculated that were the cod to derive its vitamin D preformed from the caplin, it would have to consume during 4 weeks about 26 times its weight of caplin. Although the cod does gorge itself remarkably with these little fish, it is difficult to believe that sufficient of them could be digested to supply the vitamin required. Even if there be an error of several hundred per cent in the above data, which is not probable, the *reductio ad absurdum* is still apparent. It is therefore suggested that at least a portion of the vitamin D of cod liver oil must originate by synthesis within the fish.

To investigate further the endogenous formation of vitamin D a series of piscicultural experiments was undertaken. Attempts to fatten captured codfish on a diet of veal having been unsuccessful

<sup>8</sup> About 75 years ago Fleury (8) noticed the remarkable fattening that occurs in the Newfoundland cod from June to September. Year by year the feeding conditions are much the same. The elaboration of oil is most rapid during the 1st month, while the caplin are available. Later the diet of the cod becomes heterogeneous, and the fattening relatively slower.

ful, a fresh-water fish was chosen for the following tests. Young channel catfish (*Ictalurus punctatus*) weighing about 120 gm. were removed to the laboratory from the Ohio river. Twenty of these fish were killed at once, and their visceral fat deposits extracted with cold ether. The oil thus obtained was about  $\frac{1}{8}$  as active as cod liver oil. The remaining fish were maintained in darkened aquaria on a diet of trimmed raw veal muscle. A sample of extracted veal fat exhibited no antiricketic activity when administered to rats at a level of 4.0 per cent in Diet 3143.

After 9 weeks on the vitamin-deficient diet ten of the fish were killed. They had grown slowly and were of normal appearance. Their oil assayed essentially the same as that of the twenty controls. Such continued potency could be the result of either synthesis or storage.

During the next few months several fish died of disease or accident. Only two survived, and these were killed after 6 months on the veal diet. They had doubled in weight, and their visceral fat deposits appeared to have increased considerably. The oil extracted from these fish was of normal, or slightly enhanced, potency. In evaluating this additional evidence of endogenous origin one should, of course, consider the remote possibility that the veal muscle contained undetected traces of vitamin D which the fish, with great economy, might have salvaged.

Some species of fish inhabit such shallow water that conceivably the ultra-violet rays of the sun, acting upon their skin, may occasion the formation of antiricketic material. Seven of the original fish were regularly subjected to ultra-violet irradiation under 20 cm. of water. The light source was a 220 volt Uviarc located 50 cm. above the water. The fish were irradiated in a clean aquarium for 5 minutes every other day during 6 weeks. These brief exposures profoundly affected the fish—three of them died, and the others became subnormally active, did not eat well, declined 25 per cent in weight, and lost their dorsal skin and barbels. After the 6th week they were killed, and their oil was extracted as before. Assay of this oil indicated clearly that no increase in potency followed irradiation (Table II). Inasmuch as these fish must have received far more ultra-violet energy during 6 weeks than they normally receive in a much longer period, it would seem that the vitamin D of fish oils is not produced by the action of radiant

energy. It is well to compare this work with a recent experiment by Hess and Weinstock (9), who found that the extremely potent

TABLE II.  
*Protocols of Rats Receiving Catfish Oil Preparations During 5 Days.*

Rat No.	Preparation administered.	Grade of test.	Weight.	Average daily consumption.
			gm.	gm.
2032	Oil from control catfish, 2.0 per cent.	—	55-55	4.8
2051	" " " " 2.0 " "	— (?)	55-57	6.0
2052	" " " " 2.0 " "	—	72-76	9.0
2053	" " " " 2.0 " "	—	57-62	7.8
2042	" " " " 4.0 " "	++	52-60	7.0
2047	" " " " 4.0 " "	++	80-81	8.6
2048	" " " " 4.0 " "	++	64-68	8.2
2050	" " " " 4.0 " "	—	58-60	6.8
2066	" " " " 4.0 " "	++	73-76	9.0
2247	Oil from catfish that were kept 9 wks. in dark on diet of veal muscle, 4.0 per cent.	+	66-68	5.6
2248	" " " " " "	—	62-67	6.0
2275	" " " " " "	++	58-59	6.2
2276	" " " " " "	+	55-58	6.2
2277	" " " " " "	++	78-80	7.6
2814	Oil from catfish that were kept 6 mos. in dark on diet of veal muscle, 4.0 per cent.	+++	62-68	8.4
2918	" " " " " "	+++	53-56	8.8
2246	Oil from catfish that were irradiated 6 wks. while on diet of veal muscle, 4.0 per cent.	—	75-78	6.2
2274	" " " " " "	+	52-55	6.2
3070	Veal fat control, 4.0 per cent.	—	49-50	5.8
3071	" " " " 4.0 " "	—	51-53	8.8
3072	" " " " 4.0 " "	—	46-50	8.0
3073	" " " " 4.0 " "	—	59-60	8.0
3074	" " " " 4.0 " "	—	57-61	8.0

liver oil of the puffer fish was *not diminished* in activity while the fish were protected for 3 months from ultra-violet rays and fed a

diet of herring. (Herring is a good source of vitamin D.) It is significant that the catfish, in contradistinction to cows (10), goats (11), and women (12), did not elaborate vitamin D in response to irradiation.

It is not known whether the antiricketic substance of fish oils, of irradiated mammals, of animals that have eaten antiricketic foods, and of irradiated foods and their sterols, is one and the same substance. Quite possibly vitamin D is not a single substance, but a mixture or series of substances as variable as the sterols with which it seems to be almost inseparably associated.

I am aware that the evidence supporting the theory that vitamin D is synthesized by fish is open to discussion. It leads, however, to a search for the enzymes and substrates which may be concerned in such a synthesis. Some experiments on the autolysis of fish tissues with and without added substrates have thus far proved irrelevant, but they are being continued.

#### SUMMARY.

1. Vitamin D occurs in the liver and body oils of many species of fish, but the quantity present varies widely for different species. The oil of the adult seal is slightly antiricketic, while that of the new born seal is inactive. Vegetable oils, oleo oil, veal fat, whale oil, and commercial hydrogenated cod liver oil are inactive.

2. The principal food (caplin) of the Newfoundland codfish apparently does not contain enough vitamin D to account for the vitamin accumulated by the cod during its midsummer period of fattening.

3. Piscicultural experiments demonstrated that the vitamin D of catfish oil was not increased by irradiating the fish; and it was not decreased by keeping the fish for 6 months in the dark on a vitamin-deficient diet.

4. It is suggested that the substance, or group of substances, known as vitamin D may be synthesized by fish.

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# INFLUENCE OF SALT CONTENT OF COLLOIDS UPON THEIR ELECTROMOTIVE FORCES WHICH MAY EXPLAIN BIOELECTRIC CURRENTS.

BY R. BEUTNER AND A. MENITOFF.

(From the Department of Physiology and Pharmacology, School of Medicine,  
University of Louisville, Louisville.)

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In a previous publication (1) we have shown that starch paste and other non-protein colloids afford just as satisfactory models for explaining the origin of bioelectric currents as proteins.

Hoeber's description (2) of those protein cells as the only true model for tissues should, therefore, rather be amplified so as to state that various "colloidal cells" furnish a model, the specific character of the proteins being unimportant, but further evidence tends to show that the actions observed are not so much due to the colloids themselves but rather to the water which these imbibe.

In order to prove this, measurements were performed in which a salt, preferably KCl, was added to that colloid which was used as a central conductor, such as gelatin, agar, etc. It was then observed that with such a salt addition, the electromotive forces either disappeared entirely or became very much reduced; *e.g.*, a gelatin plug between a KCl and a LiCl solution (both  $M/10$ ) produces an electromotive force of 0.012 volt, but with KCl added to that gelatin (in a concentration of  $M/2$ ) the electromotive force between the same solutions was 0.001 volt. Many more measurements of this kind were performed all proving that with an increasing salt content of the colloid, the electromotive force produced by it became smaller and eventually disappeared. The following examples may be quoted:

- KCl gelatin, no tetramethylamine HCl + 0.012 volt.  
addition
- KCl gelatin, tetramethylamine HCl + 0.000 "  
 $M/2$  KCl



+ KCl	gelatin, no addition	K <sub>2</sub> SO <sub>4</sub> -	.....0.013 volt.
+ KCl	gelatin, m/2 KCl	K <sub>2</sub> SO <sub>4</sub> -	.....0.001 "

The same is true for agar. One observation out of a large number of similar ones may be quoted: Between KCl and LiCl solutions a plug of 1 per cent agar, with no addition, gives 0.014 volt; if  $m/10$  KCl is added to the agar only 0.005 volt is observed; if a molecular KCl solution is added 0.002 volt only is seen. This influence of the salt content upon the electromotive force explains the discrepancy of the observations of different observers, *e.g.* Matsuo (3) and Mond (4). Both of these had measured electromotive forces with gelatin tannate and obtained widely different figures, although both were working in Hoeber's laboratory. Matsuo's figures were lower probably because he added a salt (NaCl) to the tannic acid solution used in preparing the gelatin tannate. Still higher electromotive forces were observed by Mond (4) and later by Deutsch (5) with serum proteins which were freed from their salt content by means of electrodialysis. One figure may be quoted from Deutsch:

— m/10 KCl euglobulin m/10  $(\text{CH}_3)_4\text{NCl}$  + 0.02 to 0.03 volt.  
(salt-free)

This value is considerably higher than those observed on agar, starch paste, and kaolin by the present writers (see previous publication); it is possible, however, to obtain higher values on agar as well, if it is freed from its natural salts.<sup>1</sup> For this purpose, a 1 per cent agar gel was partly freed from its salt content by dialysis simply by watering it in distilled water for about a week; with this agar the cell

— m/10 KCl | agar | m/10 propylamine HCl +

had an electromotive force of 0.033 volt (while with non-dialyzed agar, this was only 0.012 volt). With tetramethylamine HCl

<sup>1</sup> Samec and Ssajevitz found 4.3 per cent ash in a sample of commercial agar, one-half of which was sulfuric acid (Samec and Ssajevitz, V., *Compt. rend. Acad.*, 1921, clxxiii, 1474).

instead of propylamine HCl the same value was seen, which is even slightly larger than Deutsch's observations on euglobulin.

*All these experiments prove that electromotive forces of the kind described, arise whenever the salt solutions are bordering on a salt-free layer containing a colloid.* It is practically impossible to produce such a layer without any salt, but a layer with a low salt content will show qualitatively the same result; this we may call a "practically salt-free" layer. As all salts diffuse into the practically salt-free layer, there cannot be a sharp boundary line, as in the case of phase boundary of immiscible phases. The electromotive force of all these cells cannot be due, therefore, to phase boundary forces which were proved by one of the writers to exist on the phase boundary of water-insoluble substance, and in some cases on living tissue (6).

At the junction of a gelatin gel or an agar gel, etc., with a salt solution, the salts diffuse freely into the gel. After this diffusion has become entirely finished and equilibrium established, the salt concentration in the entire gel and in the adjacent solutions would be identical, but this would take years. In the present experiments, equilibrium is established in an exceedingly thin layer of the gels only, which is adjacent to the salt solutions. In this layer the salt concentration equals the salt concentration of the adjacent aqueous solution, but, from there on towards the interior of the gel, the salt concentration falls to practically zero. The electromotive forces observed certainly are generated along this fall of the salt concentrations. Their direction and magnitude can be calculated from the relative velocity of anionic and cationic migration of the respective salt.

According to these well known laws, the potential difference, due to the fall of the concentration, is almost zero for KCl as its ionic velocities are nearly equal (for  $K^+ = 64$ , for  $Cl^- = 65$ ) in LiCl, however, the anionic velocity is larger (the mobility of  $Li^+$  being only 35); therefore, in the fall of the concentration of LiCl, the  $Cl^-$  ions hasten on before the  $Li^+$  ions, causing a negative charge of the more dilute part of the solution, owing to their own negativity. A positive charge, therefore, is generated on the side on the LiCl solution itself. This agrees with the experimental findings. (A calculation of the exact magnitude is impossible as the salt content of the colloid is not well defined.)

Other salts in which the anionic velocity prevails are the amines, e.g.  $C_2H_7NH_2Cl$  and  $(CH_3)_4NCl$ , and in these cases also a positive potential difference is observed. Sulfates, on the other hand, have a larger cationic velocity, hence the opposite direction of the electromotive force is seen in this case.

The conclusion to be drawn from all this is that well known electrochemical laws account for the action of the protein cells; Hoeber's suggestion that rest valencies play a rôle is without foundation (it would be curious if starch paste or kaolin had rest valencies). The action of the protein cells is due to the water contained in the protein and the relative absence of salts in it. It is possible for the presence of colloids to influence ionic mobilities and in this way also the E.M.F. but this can only cause secondary variations; even this variation, if present, would in no way be peculiar to proteins.

*Remark.*—In his well known book, Loeb (7) describes cells containing gelatin as central conductors. These measurements are, however, entirely different from those of Hoeber and his collaborators because Loeb's measurements were performed *exclusively after the establishment of an equilibrium* which—owing to the presence of amphoteric colloid—is a Donnan equilibrium as clearly set forth by Loeb. Loeb's potential differences are, therefore, true phase boundary potential differences; these are constant, reversible, and definite as they can be calculated from the ionic concentrations according to the logarithmic formula of Nernst. Furthermore they are independent of ionic mobilities. None of these properties is obtainable with Hoeber's arrangements because equilibrium is *not* established, the inconstancy is illustrated by comparing the above mentioned values of Mond and Matsuo (compare the table in our previous publication (1)); there can be no reversibility as the diffusion of the salt into the salt-free layers progresses; an application of a quantitative formula like Nernst's has not been tried and seems to be impossible. The E.M.F. depends upon ionic mobility, as shown above. It is quite possible that in some of Hoeber's cells phase boundary potentials exist, but superimposed upon these are the ever changing diffusion potentials which chiefly account for the actions observed.

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## FAT-SOLUBLE VITAMINS.

### XXVII. THE QUANTITATIVE DETERMINATION OF VITAMIN A.\*

By H. STEENBOCK AND KATHARINE H. COWARD.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

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In April, 1923, Steenbock and Nelson (1), experimentally verifying observations of Hume (2) and of Goldblatt and Soames (3), advanced the theory that under certain conditions the antirachitic vitamin is essential for normal growth. They arrived at this conclusion because rats which had become stationary in weight on a diet deficient in vitamin A were made to resume growth either by exposing them to the radiations of a quartz mercury vapor lamp or by feeding them cod liver oil in which vitamin A had been destroyed by aeration. They suggested that when growth first ceased, it resulted from a deficiency of the antirachitic vitamin and not of vitamin A because ophthalmia and symptoms of the respiratory tract did not make their appearance until much later and were not influenced as to time of incidence by irradiation. With the exhaustion of this reserve of vitamin A neither radiations nor aerated cod liver oil could effect maintenance or induce growth.

They emphasized these correlations because it had been universally accepted that failure of growth on rations otherwise complete but low in fat-soluble vitamins was due to a lack of vitamin A. These conclusions attracted a great deal of attention because obviously, if correct, most of the observations on vitamin A as recorded in the literature would be in error except in those cases where by chance the animal had been furnished a sufficiency of the antirachitic factor and the very isolated cases where ophthalmia instead of growth had been used as a criterion.

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Goldblatt and Soames (4) in a second paper published by them extended the scope of their experiments and demonstrated that livers from irradiated rats were growth-promoting. Steenbock and Black (5) verified these facts and by further experimentation showed that this growth promoting property was not related to vital activity of the material exposed nor due to translocation of vitamin A. Steenbock, Black, and Nelson (6) found that it could be induced with antirachitic activation in macerated excised animal tissues; in the basal synthetic ration usually used by them for fat-soluble vitamin studies; in crude fats, and in the unsaponifiable fraction of crude fats as well. Steenbock, Black, Nelson, Nelson, and Hoppert (7) later showed that besides a considerable array of naturally occurring foodstuffs, of known compounds, only cholesterol and probably phytosterol were capable of acting in a similar capacity.

In the meantime Hess and coworkers among their other notable experiments, demonstrated the antirachitic activation of oils (8) and cholesterol (9) directly without correlating this phenomenon with the process of growth. Their experiments, in fact, closely followed in time of execution those of Steenbock and Black (5), and their announcement (9) in regard to the antirachitic activation of cholesterol was, in fact, made simultaneously with that of the latter workers (7). In Europe, entirely independently Drummond, Rosenheim, and Coward (10) showed that irradiated cholesterol could serve as a growth-promoting factor; and later Drummond, Coward, and Handy (11), accepting the demonstration from this laboratory that irradiated cholesterol promoted growth by providing the antirachitic factor, made use of irradiated cholesterol in connection with a new technique for vitamin A determination. In this technique they provided their rats with 1 mg. of irradiated cholesterol daily in addition to their fat-soluble vitamin-free diet. This allowed them to dispense with irradiation of the rats as Steenbock, Nelson, and Black (12) had advised.

As a matter of fact we had been in doubt for some time as to the permissibility of irradiating our rats, not so much when we used growth as the determining reaction but when we used change in eye condition instead. Ophthalmia as produced by vitamin A deficiency is a complex pathological reaction, and it is conceivable that the short ultra-violet rays emitted by the quartz mercury

vapor lamp may act destructively or constructively according to the sensitivity of the affected tissues and the invading microorganisms. At any rate by exposure to ultra-violet radiations there always exists the possibility that the course of the ophthalmic reaction may be changed independently of vitamin A additions. The appreciation of this possibility led to our adoption, soon after the discovery of antirachitic activation, of irradiation of the basal synthetic ration in place of irradiation of the animals.

With this modification of our vitamin A technique, Dr. E. M. Nelson and Professor Lois K. Stewart obtained very satisfactory results in the spring and summer of 1925 (13). The rats continued to grow practically up to the time of incidence of ophthalmia and sometimes even longer; and later with the addition of vitamin A they responded with growth much more promptly.

Irradiation of the basal synthetic ration accomplished the object that we were after, but a number of objections can be raised against it. In the first place with the growing tendency to feed more highly purified diets—which practice is most certainly to be encouraged—there obtains the possibility that the diet may be completely freed from activatable compounds. The probability of this at the present time appears rather remote because we have experienced no difficulty in activating alcohol-extracted and heated casein, corn-starch, or the dextrin which is prepared from corn-starch. In fact, we have found that corn-starch may be extracted with alcohol for weeks without losing its ability to become activated. Furthermore, we have always obtained very decided antirachitic response when only 5 per cent of the diet was composed of irradiated starch or dextrin. Nevertheless, we must admit that certain preparations of potato and rice starch, sugars, salts, and some pure proteins cannot be activated. Thus there always obtains the possibility that the experimenter compounding his ration from different sources may secure a diet which will not respond as expected. In the second place, it may sometimes be desirable to avoid the possibility of destruction of vitamins present in the basal diet. In our experience both vitamin A and vitamin B have shown remarkable stability to ultra-violet light. But here again we cannot be absolutely certain that these compounds are stable to ultra-violet radiations under all conditions, and therefore the use of ultra-violet light on the ration itself may be contraindicated.



Taking these objections into consideration, with a view to the needs of the future rather than the immediate present, we believe that the use of irradiated cholesterol with proper precautions is to be highly recommended for the rat. As to its use for other animals, we are not so convinced because it is not established that cholesterol is absorbed so well as the activatable compounds found in plant materials. Witness, for instance, the results obtained by Hart, Steenbock, Kletzien, and Scott (14) who found that the antirachitic unsaponifiable constituents of cod liver oil did not establish a positive calcium balance in a goat unless fed in solution in a liquid fat such as corn oil. Not only does this present concrete evidence of the variation in the assimilability of the antirachitic constituents with the diet, but it should make us very skeptical of the equivalency of antirachitic agents from different sources. It is probably not unwarranted to surmise that the antirachitic compounds which exist in the finely divided condition, often even in chemical combination, in plant tissues are much more easily assimilated than activated cholesterol administered *en masse*. In recognition of this factor we have always taken the precaution when administering cholesterol of feeding it by evaporating its ether solution directly on the basal ration. Furthermore, it is absolutely necessary to standardize each preparation of cholesterol on rachitic animals. Different preparations vary tremendously in activity, and an activity once induced is far from being a permanent property.

Whatever method of supplying the antirachitic factor is selected, we are convinced that there should be used a more specific reaction than growth to indicate whether or not vitamin A is present. As to what this should be, we are not absolutely positive because it is very evident that the absence of vitamin A from the diet leads to degenerative processes in many different organs with corresponding variation in symptoms. Tentatively, we have accepted the ophthalmic reaction and have found it to serve our purpose admirably. In the first place, in the course of the last few years, we have had ophthalmia incident in our animals on a vitamin A-free diet almost without fail. In the second place, ophthalmia is incident in the very early stages of vitamin A depletion and is readily detectable as such; and, in the third place, it responds very readily to treatment with vitamin A.

In comparison with the ophthalmic reaction, growth is so much more complex and dependent upon so many more factors, that it obviously cannot be considered in the same category. On administering a very small dose of vitamin A, we have often noted the cure of ophthalmia without resumption of growth in the following few weeks. A somewhat larger dose of vitamin A will cure ophthalmia in 10 to 14 days during which time growth will be at a standstill but will be resumed when the trouble has cleared up. A really large dose of vitamin A will result in the curing of the disease and continuation of growth at the same time. The use of growth only, as a criterion of vitamin A depletion, may have disastrous results. While waiting to be certain that growth has really ceased and that the cessation is not merely temporary, the rat may decline in weight so rapidly that recovery cannot be established even with the addition of large amounts of vitamin A. In these latter cases we have observed that failure is usually due to infections of the respiratory tract or congestion of the mesenteric vessels of the upper part of the small intestine which by its severe nature tends to terminate the animal's existence rapidly.

It is also to be noted that where only small doses of vitamin A have been administered, recovery may be only temporary, a second attack of ophthalmia developing and growth again ceasing. On the other hand, where large doses of vitamin A have been administered, the animal grows to maturity. It is evidently of the greatest importance in making comparative studies of the vitamin content of different materials, to carry on the test for long periods of time, 10 to 12 weeks generally being necessary where the amount of vitamin A administered is small.

The experimental work reported in this paper deals exclusively with the determination of vitamin A in various grains while the antirachitic factor was supplied by irradiation of the ration. We have obtained such clean cut results in this manner that we believe them worthy of presentation at this time.

#### EXPERIMENTAL.

At an age of a little more than 3 weeks when weighing from 40 to 45 gm., black and white piebald rats were given a basal ration consisting of purified casein 18 gm., agar 2, salts 40 (1) 4, dried brewer's yeast 8, and partially dextrinized starch 68.

The constituents were mixed dry—no water was added. To make it antirachitic, 100 gm. of the ration were spread over an area of 4 sq. ft. in a metal tray, and then irradiated for half an hour at a distance of 2 ft. with a BY Cooper Hewitt quartz mercury vapor lamp run at 4 amperes, 40 volts. It was stirred after 15 minutes. The irradiation was not carried out every day; a week's or even a month's supply was prepared at a time because the activation induced is a very stable property.

The members of one litter were kept together in a large cage ( $2' \times 2' \times 1\frac{3}{4}'$ ) on wire screens during the preparatory period, but in separate cages during the test period. They were weighed individually once a week for the first 3 weeks, then 2 or 3 times per week. Careful examination for the onset of ophthalmia or respiratory disturbances was made daily and it was found that such trouble was definitely established generally within 5 weeks of the beginning of the experiment and within 3 or 4 days of the appearance of the first signs. It is significant that all the members of any one litter generally developed ophthalmia within a very few days of each other.

#### *Vitamin A Content of Grains.*

In illustration of this technique may be quoted some experiments carried out with various grains as the source of vitamin A. Recent experiments on the vitamin A content of yellow corn seeds and seedlings had suggested that yellow corn contained more vitamin A than had previously been suspected. In order to confirm this, one rat from a litter showing definite signs of depletion of vitamin A was given one yellow corn seed per day, two others were given two yellow corn seeds, while two others were given four yellow corn seeds. The rat on one seed recovered completely and grew for a time but eventually declined in weight and died (Chart I). The rats on two seeds also recovered and grew for a longer period of time but eventually died also; while those on four seeds grew to maturity though eventually they too developed respiratory trouble. The experiment was terminated before they died. Reproduction was not attempted. Consumption records showing what percentage of the total food intake the corn seeds constituted in the later stages of the experiment are shown in Table I.

A further experiment was carried out a little later than the above in order to determine what percentage of the total food intake the corn formed during the early weeks of the experiment. Three rats from one litter were given one yellow corn seed each per day

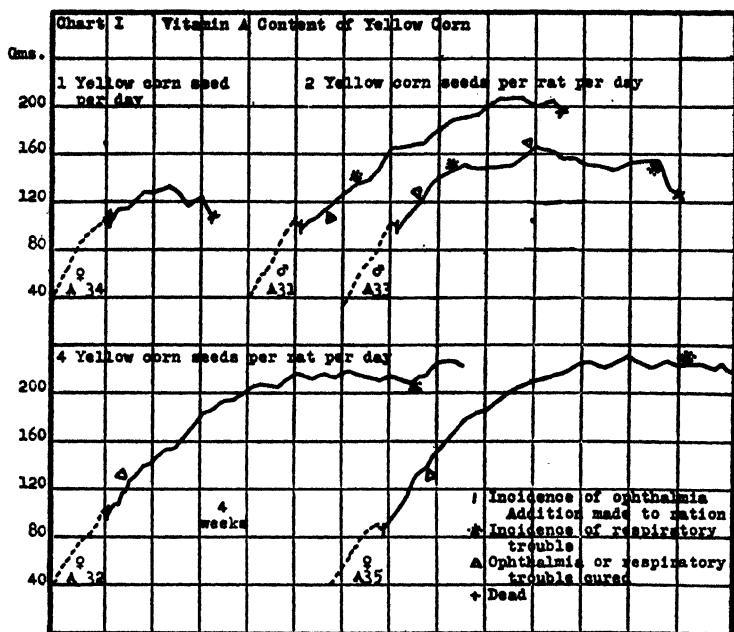


CHART I. All the rats in this experiment were from the same litter and all gave a response to yellow corn seed additions. In Rat 34, receiving one yellow corn seed daily, the eye symptoms improved temporarily but became worse later and the rat was never cured. In Rats 31 and 33, each receiving two yellow corn seeds daily, the eye symptoms were cleared up quickly, and growth was resumed for a considerable period of time. The rats did not reach maturity, however, and eventually died of some form of respiratory trouble. In Rats 32 and 35, each receiving four yellow corn seeds daily, the eye symptoms cleared up quickly, growth was more nearly normal than in the rats on two corn seeds, and duration of life was longer.

and consumption records were taken daily (Chart II). For a week's ration for each rat, seven seeds were chosen weighing altogether 2.5 gm., and any one of the seven seeds was given to the rat daily. This constituted some 5 per cent of the total food intake at the beginning of the experiment but a smaller percentage

as the intake increased with the growth of the rat (Table II). The effectiveness of the low dosage of yellow corn in curing ophthalmia and, in the rather higher dosage, promoting normal growth when added to a basal ration made definitely antirachitic by irradiation, indicates that yellow corn is comparatively rich in vitamin A; and the fact that previously 85 per cent had been

TABLE I.  
*Food Intake of Rats A 31, 33, 32, 35 (Chart I) during the 14th to 18th Weeks of the Test Period.*

Rat No.	Average body weight for period.	Total food intake.	Total weight of corn seeds eaten.	Yellow corn seeds per cent of total food intake.
	gm.	gm.	gm.	
A 31	196	232	25	7.5
" 33	155	258	25	9.7
" 32	213	375	50	13.2
" 35	216	382	50	13.0

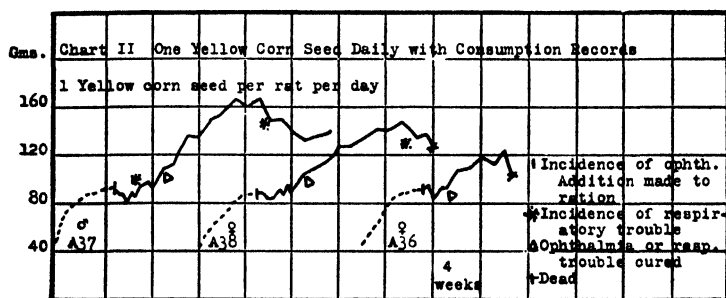


CHART II. All the rats in this experiment were from the same litter. Ophthalmia was cured and growth was promoted temporarily in each case. In Rat 37, ophthalmia and the first attack of respiratory trouble were cured about the same time.

found necessary in a diet which was almost certainly not antirachitic (15), shows that yellow corn is remarkably poor in the antirachitic factor and that its previous failure to promote growth was due to a deficiency of the latter factor rather than of vitamin A. A similar vitamin content of hog millet has been recorded by Steenbock, Nelson, and Black (12).

It was considered desirable at this point to make a comparison between yellow and white corn on the new basal diet; that is, a definitely antirachitic one. One test was made using one yellow corn seed to four white ones; another was made using four yellow corn seeds to four white ones. The dosage of four white corn seeds was in each case totally inadequate to indicate even traces of vitamin A, while the results obtained with one or four yellow corn seeds confirmed the previous ones (Chart III). The yellow and white seeds were of approximately equal size and weight.

TABLE II.

*Food Intake of Rats A 37, 38, 36 (Chart II) during Each Month of the Test Period, Compiled from Daily Consumption Records.*

Rat No.	Average body weight.	Total food intake.	Total weight of corn seeds eaten.	Yellow corn seeds per cent of total food intake.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
A 37	99	220	10	4.5
	145	250	10	4.0
	161	203	10	5.0
	140	233	12.5	5.4
		5 wks.		
A 38	95	190	10	5.3
	135	239	10	4.2
	160	200	10	5.0
	130	89	5	5.6
		2 wks.		
A 36	103	177	10	5.6
	112	125	7.5	6.0
		3 wks.		

An attempt was then made to discover whether the vitamin A was concentrated in the endosperm or in the embryo of the yellow corn seed. The embryo of one seed gave no indication of the presence of vitamin A, nor did a daily dosage of sixteen embryos, a weight equal to that of the endosperm of an average seed. The endosperm of one seed, however, indicated the presence of vitamin A. Hence it may be concluded that the vitamin is largely, if not entirely, concentrated in the endosperm of the yellow corn seed (Chart IV). This confirms previous unpublished experiments.

Wheat embryo has been reported almost from the time of beginning of vitamin research (16) as containing small though definite amounts of vitamin A, and it now appeared conceivable that in this case also, a lack of the antirachitic vitamin rather than a lack of vitamin A might be responsible for this conclusion. Wheat embryo (a representative commercial sample obtained from and selected by one of our largest milling companies as being of excellent quality) was tested by adding it to the irradiated basal diet to

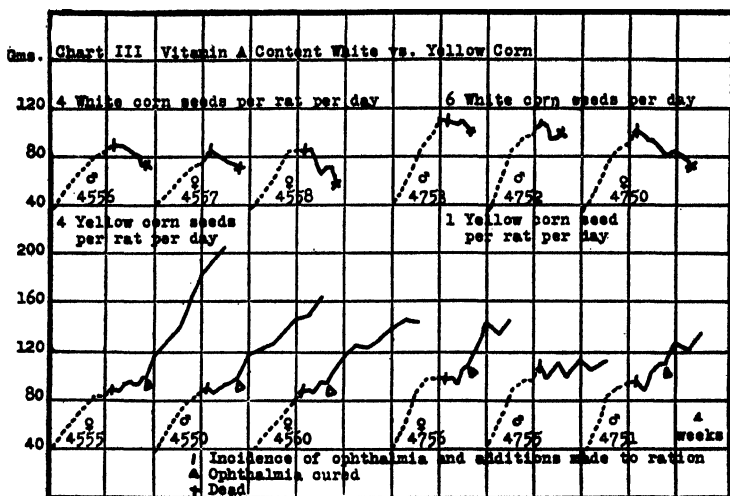


CHART III. Rats 4555 to 4560 were from one litter, Rats 4750 to 4755 from another. There was no improvement in the condition of the rats on four white corn seeds. The rats on one yellow corn seed (except Rat 4755) recovered quickly and even resumed growth. Those on four yellow corn seeds recovered quickly also and grew more steadily and quickly than those on only one.

the extent of (a) 10 per cent, and (b) 20 per cent of the ration. 10 per cent furnished some indication of the presence of vitamin A, while 20 per cent gave much more definite evidence, one rat growing on this amount remarkably well (Chart V). But it must be remembered when evaluating the nutritive properties of the entire wheat kernel that embryo constitutes about 1.5 per cent of the dry weight of a whole wheat grain (17) and that, therefore, 20 per cent of this sample (which admittedly was far from being pure embryo)

would represent an amount of whole grain equivalent to more than 13 times the entire ration. Actually the amount was far less than this because the sample of germ was not pure.

A further comparison was made between whole grains of wheat, oats, and white corn using our basal synthetic ration as a control.

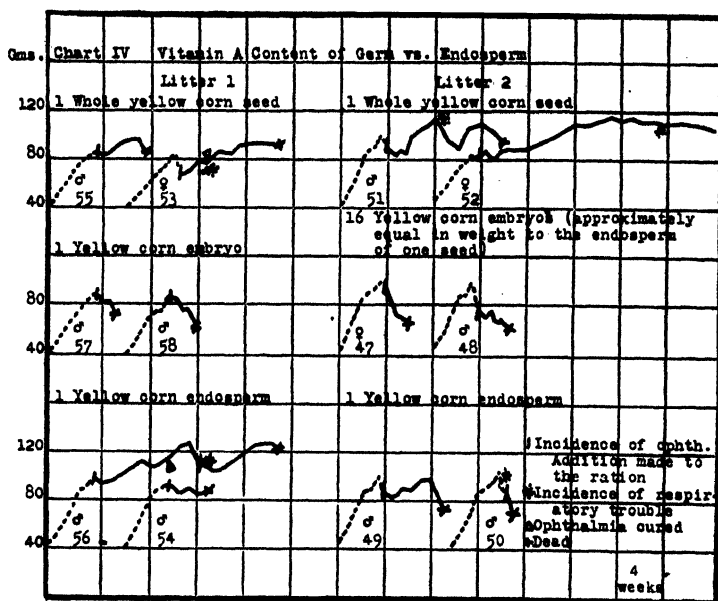


CHART IV. Rats 53 to 58 were from one litter, Rats 47 to 52 from another. There was definite though uneven improvement in the general condition of the rats on the whole yellow corn seed and of those on the corn endosperm, except in Rat 50 which was probably in too bad a condition for the experiment when the change in diet was made. There was no improvement at all in the rats on the embryo only, whether one embryo was fed or sixteen embryos. In Rat 52, the ophthalmia was never cured, though the rat's duration of life was much prolonged.

Each of the grains was ground up in a mill, made up into a diet similar to the basal synthetic ration, substituting 68 per cent of the ground grain for the 68 per cent dextrin. The diet was irradiated as needed. To eliminate differences due to variations in the appetite of the rats, the possible consumption was limited to 5 gm. per rat per day during the 1st week. As even this small



amount was not eaten by all the rats, it was later decreased to 4 gm. during the 2nd and succeeding weeks. By the end of the 3rd week, only 2 rats had not eaten *in toto* as much as the others, and these were on the basal synthetic control ration. To eliminate individual differences as much as possible there were used four rats, one from each of four litters divided into four comparable groups (Chart VI). They were 23 to 27 days old and weighed 41 to 49 gm.

All four rats on the basal synthetic ration developed ophthalmia permanently in 6 to 6½ weeks; those on the wheat diet showed some

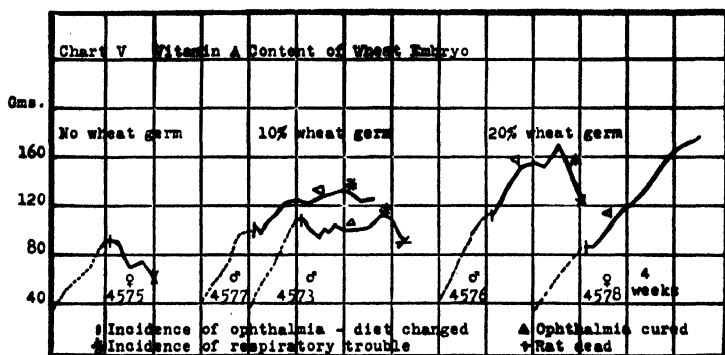


CHART V. All the rats were from the same litter. Rats 4577 and 4573, receiving 10 per cent wheat embryo, show some evidence of the presence of vitamin A; Rats 4576 and 4578, receiving 20 per cent wheat embryo, gave more definite evidence of the presence of vitamin A.

signs of it in 6 weeks, but Rats 195 and 196 recovered during the 7th and 8th weeks to succumb again shortly afterwards. Those on the oats diet developed ophthalmia in 5½ to 6½ weeks—two of these dying within the next few days of some respiratory trouble and a third in 10 days with slight hemorrhage of the stomach. On the white corn diet, 3 developed ophthalmia in 6 to 6½ weeks (one of these dying in the next 10 days); while one rat had not developed ophthalmia at the end of 8 weeks.

Apparently all these grains are very poor in vitamin A, but of the three, wheat appears to be the richest; however, for conclusive evaluation further experiments need to be performed.

The data as at present available are presented primarily to indicate the applicability of the technique to the study of these relations.

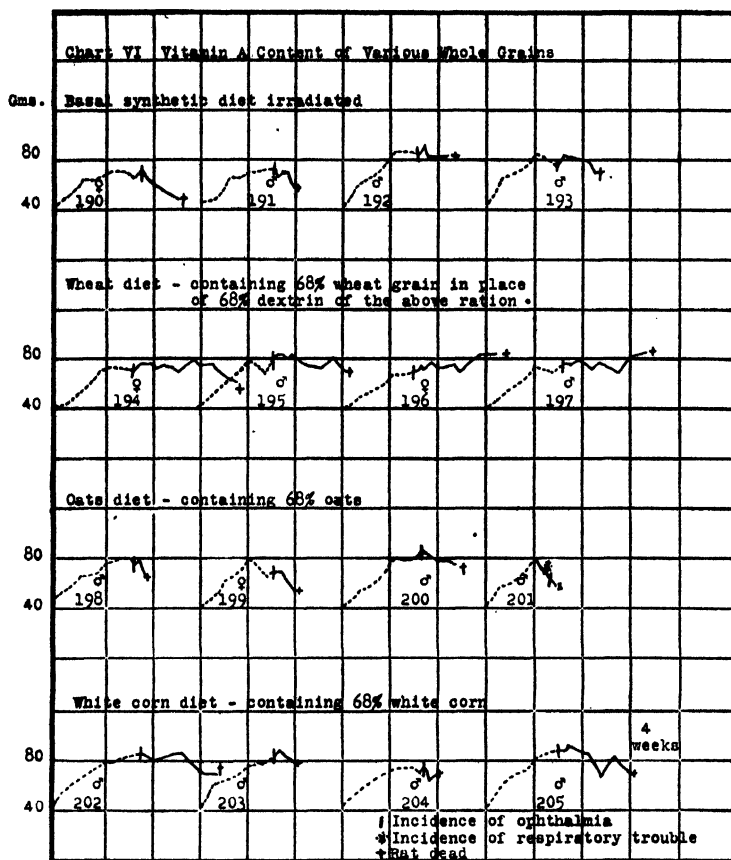


CHART VI. Rats 190, 194, 198, 202 were from one litter, Rats 191, 195, 199, 203 from another, Rats 192, 196, 200, 204 from another, and Rats 193, 197, 201, 205 from still another. All the rats on the basal synthetic diet developed ophthalmia permanently in 6 to 6½ weeks; those on the wheat diet developed faint signs of it at the same time but a remission occurred in the case of Rats 195 and 196 in the 7th and 8th weeks for about a week in each case; those on the oats diet developed ophthalmia in 6 weeks and three died in 6 to 7 weeks; all those except one on the white corn diet developed ophthalmia in 6 to 7 weeks, two of them dying in 8 weeks.

## SUMMARY.

A method of testing substances for vitamin A is described in which the antirachitic factor is supplied by irradiation of all or part of the basal diet.

Excellent results were obtained by this method but in view of the desirability of feeding synthetic diets of highly purified known constituents the advantages of feeding irradiated cholesterol in place of irradiating the ration have been pointed out.

The use of the incidence of ophthalmia is advocated as a sign of exhaustion of the animal's store of vitamin A in preference to cessation of growth. The two are often simultaneous, but the use of the former criterion prevents loss of animals through the very sudden and rapid decline that may ensue while one is waiting to become certain that growth has really ceased.

Growth ceases during the worst stages of ophthalmia and is only resumed when definite improvement in the animal's condition is observable.

Yellow corn seeds were used to demonstrate the technique described and it is to be noted that they appear to be richer in vitamin A than was previously supposed, but presumably they are poor in the antirachitic factor. Vitamin A is located in the endosperm rather than in the embryo. Commercial wheat germ when fed as 20 per cent of the diet furnishes definite evidence of the presence of vitamin A.

Whole wheat, white corn, and oats were all found poor in vitamin A, but of the three wheat appeared to contain the most. Whether these relations hold true generally remains to be established.

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## THE INFLUENCE OF LIGHT AND HEAT ON THE FORMATION OF VITAMIN A IN PLANT TISSUES.\*

BY KATHARINE H. COWARD.

*(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)*

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The influence of light on the formation of the vitamins in plant tissues has been studied very little except perhaps in relation to vitamin A. The increase in the amount of vitamin C in seeds during germination indicates that light is not necessary for the formation of this factor. If we can accept the theory of the synthesis of vitamin B by yeast, the fact that it grows as well in the dark as in the light indicates that vitamin B also can be formed independently of light, but whether light would accelerate the formation of these factors does not seem to have been investigated. Sunlight apparently has a very strong influence on the formation of vitamin A; but no limit has been reached experimentally beyond which the amount of illumination has seemed to be deleterious or even in excess of that required for maximum formation of the factor. It was with a view to obtaining information on this point that the present work was undertaken.

Coward and Drummond (1) showed that whole wheat seeds forming 10 per cent of the diet of a rat failed to bring about resumption of growth when this had ceased on a shortage of vitamin A. Coward (2) had found in 1923 that one, two, or three etiolated wheat shoots had no power to do this either, and again in 1925 (3) and in 1926 (4) had obtained the same result, and had concluded that etiolated shoots contained only traces of vitamin A or none at all. On the other hand, Wilson (5) had found that 5 per cent of dried etiolated wheat shoots in the diet of rats furnished enough vitamin A for adequate growth in the prophylactic type

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of experiment and 8 per cent of similar material enough to indicate the presence of vitamin A in a curative experiment. Harrow and Krasnow (6) reported that germinated white corn, soaked overnight and then kept in moist sphagnum in the incubator for about 20 hours, showed no higher vitamin content than the ungerminated. Later (7), in dealing with germinated and green shoots they found that the latter were much richer in vitamin A than the former, only they realized that in using equal weights of material they had used a greater number of shoots than of seeds and hence gave their result with some reserve. Stepp (8) used seedlings 3 to 4 days old. When dried, he found that they contained negligible amounts of vitamin A. When tested in the fresh state (1.5 gm. per day), he found that the eye condition of the rats improved but no growth resulted and there was no prolongation of life; but he concluded that etiolated shoots contained small quantities of vitamin A.

Electric light has been used also for the production of vitamin A in seedlings. Coward (2) produced vitamin A in sunflower shoots by subjecting etiolated seedlings, 9 days old, to illumination from an electric light of 32 candle power at a distance of 16 inches for 10 hours a day for 3 successive days. Later (4), she found that wheat seedlings, grown for 9 days in the dark, and illumined by a lamp of even 100 watts for 24 hours consecutively, were less effective in promoting growth in rats on a diet deficient in vitamin A than the sunflower seedlings treated as above. She also found (4) that wheat seedlings do not respond to sunlight as vigorously as bean or sunflower seedlings and hence adopted wheat for the present series of experiments which at first had to deal with minimal doses of the vitamin.

In order to obtain what might be considered excessive illumination of the plants, a quartz mercury vapor lamp, the Cooper Hewitt type, was used. It was run on an alternating current with a burner voltage of 50 and 4.5 amperes current density. The plant pots containing the seedlings (grown in complete darkness) were set upright on a table 24 inches from the tube of the lamp. In those experiments where the shoots were to be protected from the shorter ultra-violet rays, a Mason glass jar was inverted over the shoots, resting on the soil of the pot. Examination by means of a Hilger monochromator with a Coblentz thermopile

showed that the glass of these jars cut out all the rays of wavelength  $313\text{ m}\mu$  and from 50 to 70 per cent of rays of  $365\text{ m}\mu$ .

#### EXPERIMENTAL.

The method adopted for testing for vitamin A has been fully described by Steenbock and Coward (9). The basal diet consisted of:

Casein (alcohol-extracted and heated).....	gm.	18
Agar.....		2
Salt mixture 40.....		4
Yeast (dried).....		8
Dextrin.....		68

The salt mixture consisted of:

NaCl.....	gm.	23.36	$\text{Ca}_2\text{H}_2(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ .....	gm.	68.8
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....		24.6	Ca lactate $\cdot 5\text{H}_2\text{O}$ .....		15.4
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ .....		35.8	Iron citrate $\cdot 6\text{H}_2\text{O}$ .....		5.98
$\text{K}_2\text{HPO}_4$ .....		69.6	KI.....		0.16

The whole ration was irradiated in order to produce in it ample supplies of the antirachitic factor (10, 11). Approximately 200 gm. of the diet were spread over an area of 4 sq. ft. in a shallow zinc tray, and four of these trays were irradiated at one time for half an hour under a Cooper Hewitt quartz mercury vapor lamp running with a burner voltage of 40 and a current density of 4 amperes, at a distance of 22 inches. The diet was stirred after 15 minutes irradiation. The antirachitic properties of such a ration are retained for several weeks.

The test rats were put on this diet when 40 to 45 gm. in weight and were kept on screens throughout the experiment. In about 5 weeks, the first symptoms of ophthalmia began to manifest themselves; the eyelids became bare and swollen, the eyes watery and dirty. In about 4 days one could consider this condition definitely established and put the animal on the material to be tested for vitamin A. Growth was maintained by the rats up to this point or very nearly, but this fact is worth emphasis—if, at this stage (ophthalmia definitely started) the rats are given no supplement or a supplement containing no vitamin A, growth ceases if it has not



already done so and in a very short time there is a decline in weight and death ensues (12-14). The eye conditions also become much worse, the eyeballs themselves becoming purulent and the lids bleeding. There is total loss of sight. If, on the other hand, a supplement containing relatively small amounts of vitamin A is given to a rat with ophthalmia established, the eye condition shows definite improvement in 5 to 7 days and may be practically cleared up in 10 to 14 days. Growth during this period is generally stationary but is resumed almost as soon as a definite improvement in the rat's condition can be detected. If the supplement contains relatively large amounts of vitamin A, growth may go on simultaneously with the improvement in the eye condition.

It is remarkable that in nearly every litter of rats used (and each comparison was carried out on the members of *one* litter) the number of days required for the development of ophthalmia varied very little. This must be attributed to the previous diet of the animals, the stock colony of this laboratory having been fed on a diet as nearly uniform as possible, for many years (15).

All the plant materials tested were given to the animals fresh each day and in order to have shoots of the same age each day, seeds were set daily in flower pots in series and used at the required age. Illumination was always carried out on the plants while they were still growing in the pots.

The first point that had to be investigated was the efficiency of the quartz mercury vapor lamp as a source of light suitable for the formation of vitamin A in plant tissues. As electric light and even sunlight had been found to be rather feeble for this purpose, the first tests on (a) wheat seeds, (b) etiolated wheat shoots about 5 inches high, and (c) similar etiolated shoots irradiated 1 hour per day, immediately before feeding, were run at a comparatively high level; that is, five seeds or shoots were used in each test.

As expected, the rats receiving five wheat seeds (total 0.17 gm.) per day failed to give any response to indicate the presence of vitamin A. But it was surprising that five etiolated wheat shoots (total dry weight 0.09 gm.) 16 days old, definitely cured ophthalmia and brought about very marked resumption of growth. There had been no indication whatsoever in earlier experiments that the dosage of three shoots had been so near the margin of efficacy. The irradiation of similar shoots for 1 hour per day under

the quartz mercury vapor lamp appeared to cause the formation of further small amounts of vitamin A as indicated by somewhat more rapid clearing up of the eye symptoms and by better growth response when shoots thus treated were given to the test animals (Chart I).

A parallel test on (a) yellow corn and (b) white corn was also run, though in this case the whole seedlings (that is, root, shoot, and remains of seed) were compared. One yellow corn seed was given to each of two rats of one litter per day, one whole yellow corn seedling (average height 12 to 15 cm., average dry weight about 0.25 gm., 8 days old) to each of two others, and one seedling

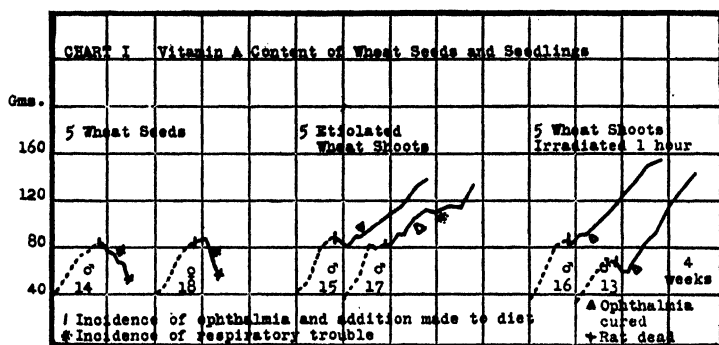


CHART I. All the rats were from one litter. No evidence was obtained of the presence of vitamin A in wheat seeds, but apparently etiolated seedlings contain small amounts of this factor and similar seedlings irradiated for 1 hour contain rather larger amounts.

grown as the above but irradiated for 1 hour, to each of two other rats. This dosage was determined by the previous finding of Steenbock and Boutwell (16) that yellow corn had to be fed as 85 per cent of the diet in order to produce normal growth in rats. It was thought that one yellow corn seed would be entirely ineffective in indicating the presence of the factor and that the shoots might show an increase; but again to our surprise, one yellow corn seed daily was found to be adequate for the curing of ophthalmia and for promoting growth for a time at least. Food consumption records revealed the fact that one yellow corn seed per day roughly constituted 5 per cent of the diet of the rat at this time, a marked contrast to the 85 per cent previously found necessary and which

is now interpreted, not as the percentage required to furnish adequate amounts of vitamin A, but as that required to furnish

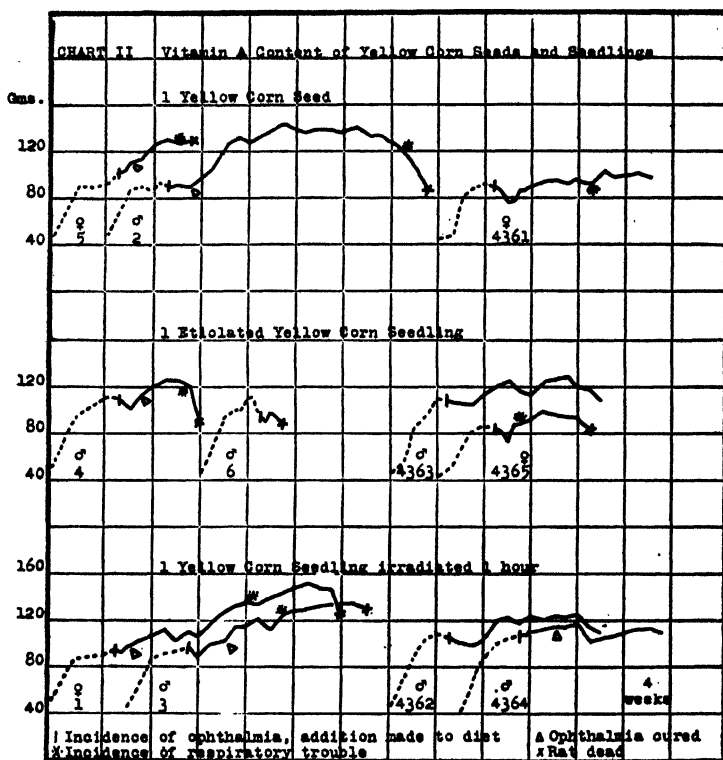


CHART II. Rats 1 to 6 were from one litter, Rats 4361 to 4365 were from another litter. Rats 5, 2, 4361 show that yellow corn seeds contain appreciable amounts of vitamin A. Rats 4, 4363, 4365 show that yellow corn seedlings also contain vitamin A. Rat 6 was apparently in too bad condition when put on experiment to give a response to the small amount of vitamin present in the seedling. Rats 1, 3, 4362, 4364 show that the irradiated seedlings may contain slightly larger amounts of vitamin A than do the non-irradiated seedlings. In Rats 4361, 4363, 4365, 4362 there was practically no improvement in the eye condition, although life was considerably prolonged.

enough of the antirachitic factor, the vitamin A thus being in excess of the amount required. In other words, the low content of the antirachitic factor in the yellow corn was the limiting

factor and when this was supplied in liberal amounts by irradiating the basal diet, the vitamin A could then be more accurately estimated. This result should be compared with that on hog millet recorded by Steenbock, Nelson, and Black (17).

The etiolated yellow corn seedlings showed very similar vitamin A content, but the seeds from which the plants had grown and which were also eaten by the rats were not nearly exhausted and probably still contained vitamin A. It is not yet known whether vitamin A can be transported, or not, and it has been shown (3) that the vitamin is not used up in any process carried on by the plant in the dark. Thus the response given by the rats to the whole seedlings may have been due to the stores of vitamin A originally present in the seeds (Chart II). An examination of the shoots only was made later (Chart IV). The irradiated seedlings appeared to have developed very little, if any, extra vitamin and it was concluded that 1 hour's irradiation had very little effect on the vitamin content of the shoots.

White corn gave more definite evidence of the formation of vitamin A on exposure to the light from the quartz mercury vapor lamp. One seed per rat per day produced no response at all. There was no amelioration of the eye symptoms and in each case, there was a rapid decline in weight and then death. With the etiolated seedlings of similar size and weight to the yellow corn seedlings, there was very little improvement in the eye condition of any of the rats but the decline in weight was much more gradual and life was definitely prolonged. Where the seedling had been irradiated for 1 hour, eye symptoms improved in one case and weight was maintained for 6 to 9 weeks before decline set in, followed by ultimate death (Chart III). In a further experiment using the same quantities of material, but irradiating for 4 hours instead of 1, similar results were obtained with the seeds and etiolated seedlings, but the seedlings irradiated for 4 hours gave definite resumption of growth before decline and ultimate death (Chart III).

A further test with etiolated *shoots* only was made in order to eliminate the effect of the vitamin A content of the seed of yellow corn. The dosage was based on the dry weight of the material. It was found that one yellow or one white corn shoot as used had a dry weight roughly equal to that of four wheat shoots (0.6 gm.

of corn shoot, fresh weight = 0.4 gm. of wheat shoots, fresh weight). The three kinds of shoots, wheat, white corn, and yellow corn gave very nearly similar results, the wheat and yellow corn being rather more potent than the white corn, but all giving

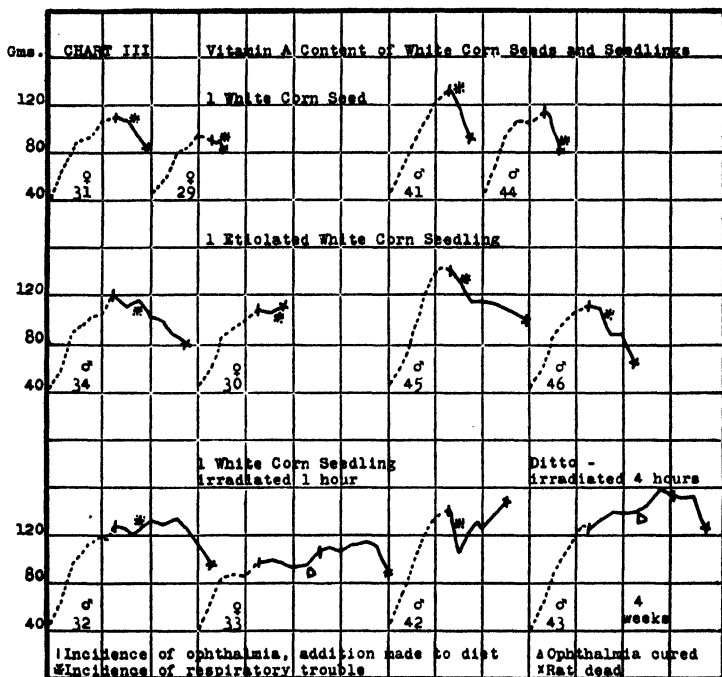


CHART III. Rats 29 to 34 were from one litter, Rats 41 to 46 were from another litter. Rats 31, 29, 41, 44 show no evidence of vitamin A in white corn seed. Rats 34, 30, 45, 46 show that there may be a slight increase in the etiolated seedlings. Rats 32, 33 show some increase in vitamin content by irradiation for 1 hour. Rats 42, 43 show a somewhat more marked effect by irradiation of the seedlings for 4 hours. Note the second attack of ophthalmia in Rats 33 and 43.

evidence of the presence of small amounts only of vitamin A (Chart IV).

In the case of the wheat, the point was carried still further. The etiolated shoots used had grown for 16 days in these experiments and the first foliage leaf had in each case burst from the

sheath and developed lipochromes. It was decided to test an earlier stage in the plant's development, so two rats from each of two litters were given five seeds each daily, two others were given five seedlings that had germinated in damp earth for 2 days and had developed a root system of three rootlets, the middle one being 1 to 15 cm. long; and two other rats were given five seedlings

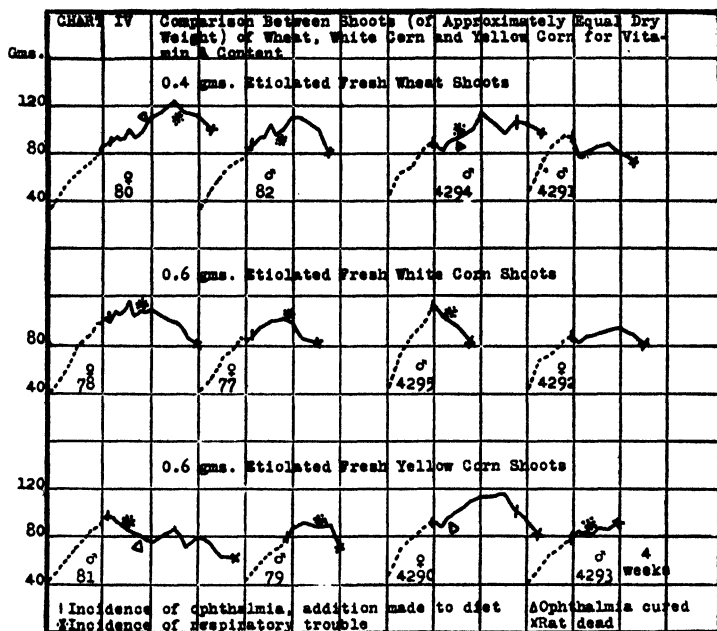


CHART IV. Rats 77 to 82 were from one litter, Rats 4290 to 4295 from another litter. The several groups on etiolated wheat shoots, white corn shoots, and yellow corn shoots fed in amounts calculated on a dry weight basis give somewhat similar results, those on the white corn shoots being rather inferior to those on the wheat or those on the yellow corn. Note the second attack of ophthalmia in Rat 4290.

(shoot, roots, and remains of seed) that had grown for 7 days and had produced well developed root systems and shoots about 12 cm. long. The plants for this experiment were grown at a temperature of 78° which was higher than that in which the plants had been grown earlier. Here, again, no evidence of vitamin A was found in the wheat seeds, neither was any found in the seedlings

of 2 days growth in spite of their greater succulency through soaking and therefore possibly greater digestibility; but those of 7 days growth gave evidence of definite amounts of vitamin A, in the amelioration of eye symptoms and well marked prolongation of life (Chart V).

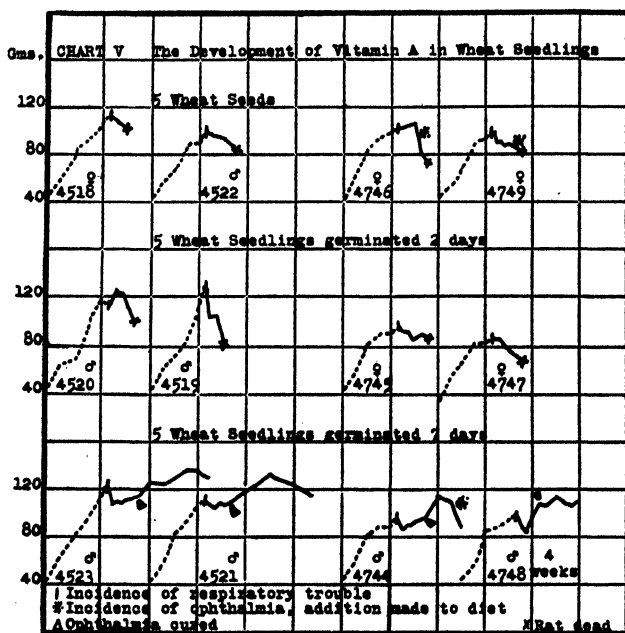


CHART V. Rats 4518 to 4523 were from one litter, Rats 4744 to 4749 from another litter. Rats 4518, 4522, 4746, 4749 receiving five wheat seeds each per day show no evidence of vitamin A in wheat seeds. Rats 4520, 4745, 4747, 4519 receiving five seedlings each per day show no evidence of vitamin A in seedlings 2 days old. Rats 4523, 4521, 4744, 4748 receiving five seedlings each per day show evidence of appreciable amounts of vitamin A in wheat seedlings grown for 7 days in the dark.

These experiments clearly indicated that it was impossible to use five wheat shoots as the dosage for a test of the influence of light on the formation of vitamin A. A comparison was then made between (a) two non-irradiated wheat shoots and (b) two wheat shoots irradiated for 1 hour. Under these conditions the influence of light was demonstrable because two non-irradiated wheat shoots

showed no evidence of the vitamin while two irradiated for 1 hour gave evidence of definite though small amounts of that factor. To emphasize this difference wheat seedlings were irradiated for 1 hour on each of 4 successive days, making a total of 4 hours irradiation in all instead of 1. When submitted to test, controls of two non-irradiated shoots gave no response, whereas those irradiated for 4 hours cured ophthalmia and prolonged the life of the

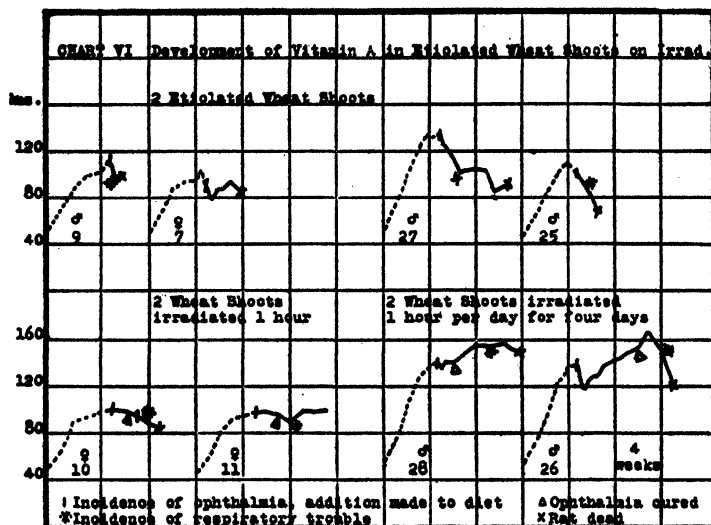


CHART VI. Rats 7 to 11 were from one litter, Rats 25 to 28 from another litter. Rats 9, 7, 27, 25 receiving two shoots each per day show no evidence of the presence of vitamin A in etiolated wheat seedlings. Rats 10, 11, 28, 26 receiving two shoots each per day give definite evidence of the presence of vitamin A in wheat seedlings which have been irradiated for 1 hour just before being given to the rats.

rats and even induced a certain amount of growth before the final decline and death (Chart VI).

In connection with this longer irradiation of seedlings for the production of vitamin A, it seemed of interest to determine whether irradiation for 4 hours continuously would have the same effect as irradiation for 1 hour per day on 4 consecutive days. Coward (3) has shown that vitamin A is not used up in any process carried on by the plant in the dark but it seemed possible that some reaction in which vitamin A was used up might be carried more



nearly to completion by 4 consecutive hours exposure to light than by 4 separate hours exposure at intervals of 1 day. Accordingly a test was carried out with (a) one wheat shoot and (b) two wheat shoots exposed under these conditions. Apparently the difference in methods of exposure made no difference in the vitamin A content of the shoots (Chart VII).

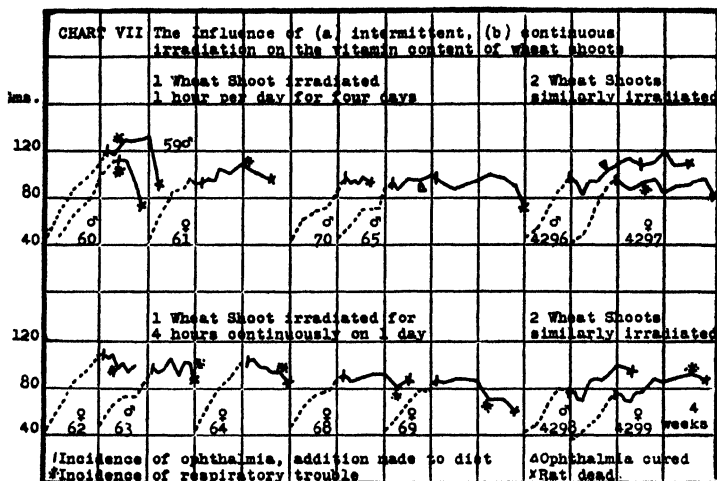


CHART VII. Rats 59 to 64 were from one litter, Rats 65 to 70 from another, and Rats 4296 to 4299 from still another litter. Rats 59, 60, 61, 70, 65 receiving one wheat shoot irradiated for 1 hour per day for 4 days showed little evidence of the presence of vitamin A in these shoots. Rats 62, 63, 64, 68, 69 receiving one wheat shoot irradiated for 4 hours continuously on 1 day showed a similar very low content of vitamin A. Rats 4296, 4297 receiving two wheat shoots irradiated intermittently as above gave rather more evidence of the presence of vitamin A. Rats 4298, 4299 receiving two wheat shoots irradiated continuously as above gave evidence of amounts of vitamin A in these shoots very similar to those irradiated intermittently for the same total length of time.

The next point to decide was whether the results obtained were due to the visible and the long ultra-violet rays or the short rays from the lamp. Coward (2) had shown that ultra-violet rays such as are absorbed by window glass are not *necessary* for the formation of vitamin A, but it was still conceivable that they might have an accelerating influence on its formation or, on the contrary, might hinder its formation. Hence experiments were made on

wheat seedlings (7 days old) exposed (a) directly to the rays from the lamp and (b) under cover of a thick glass jar that fitted inside the flower pot. This glass cut off all rays of  $313\text{ m}\mu$  and 50 to 70 per cent of rays  $365\text{ m}\mu$ . Two wheat shoots per rat per day were used for the test for the first experiment. They were irradiated for 1 hour (Chart VIII). At this level, cutting off the

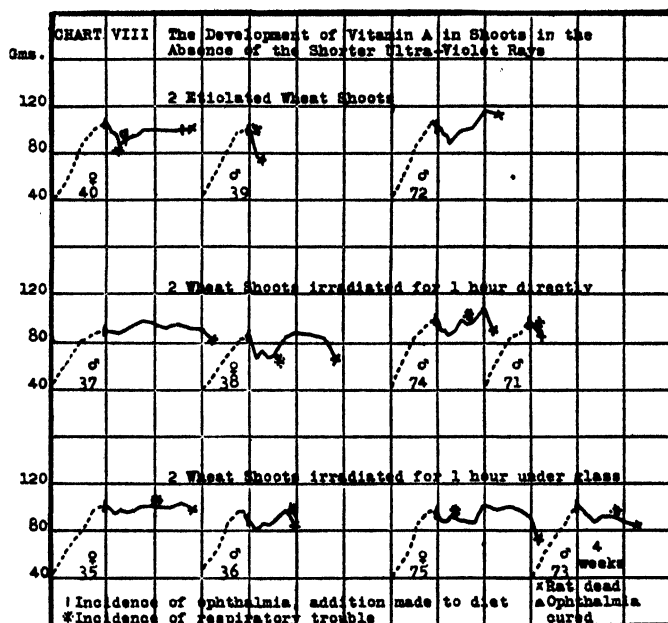


CHART VIII. Rats 35 to 40 were from one litter, Rats 71 to 75 were from another litter. Rats 40, 39, 72 receiving two wheat shoots each per day show very little evidence of the presence of vitamin A in etiolated wheat shoots. Rats 37, 38, 74, 71 receiving two wheat shoots irradiated directly, and Rats 35, 36, 75, 73 receiving two wheat shoots irradiated under glass show that the shorter ultra-violet rays have apparently no influence on the formation of vitamin A in living plant tissue.

shorter ultra-violet rays by the glass apparently had no effect, beneficial or otherwise. As these short periods of irradiation had in no case produced normal growth in the animal, weight being maintained only, seedlings were next irradiated for 4 hours per day for 4 consecutive days (a) directly and (b) under glass and fed at the same level of two shoots per rat per day. This resulted

in practically normal growth in nearly every case whether irradiation was made directly or under glass. Certainly, if this irradiation *had* been more than was necessary for maximum formation of the vitamin, it had had no deleterious effect (Chart IX). A further test still was carried out using irradiation of 8 hours per day for 5 consecutive days. It was thought that if a

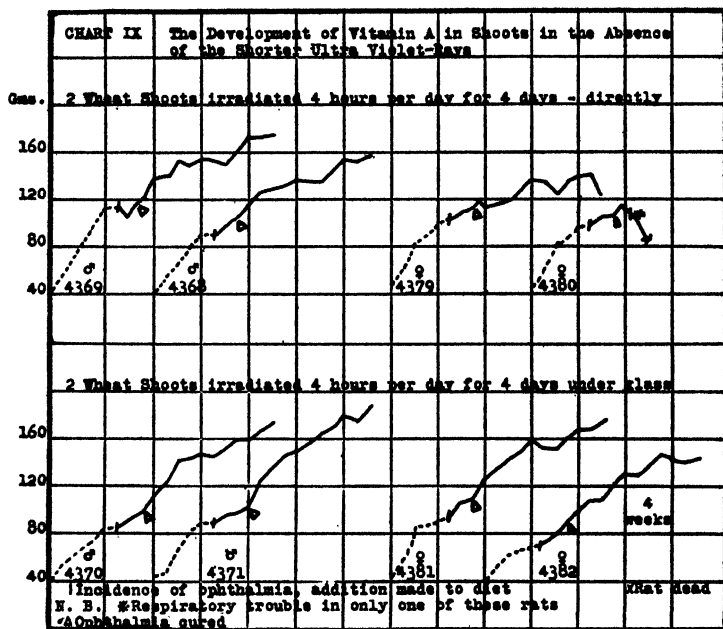


CHART IX. Rats 4368 to 4371 were from one litter, Rats 4379 to 4382 were from another litter. The very similar response given by all these rats to two shoots irradiated directly or under glass shows that the shorter ultra-violet rays have apparently no influence on the formation of vitamin A in living plant tissue.

total irradiation of 16 hours were the optimum for vitamin A formation, 40 hours would be an excessive amount. In this test again, two wheat shoots were given to each rat per day, and in each group a control was run of two wheat shoots irradiated for 4 hours on 4 consecutive days (Chart X). In each of the two litters used for the experiment, the response given by the animals was very similar, whether the irradiation had been carried on for

16 hours or for 40, directly or under glass. It seems evident, therefore, that the shorter ultra-violet rays are without influence on the formation of vitamin A in living plant tissue unless, of

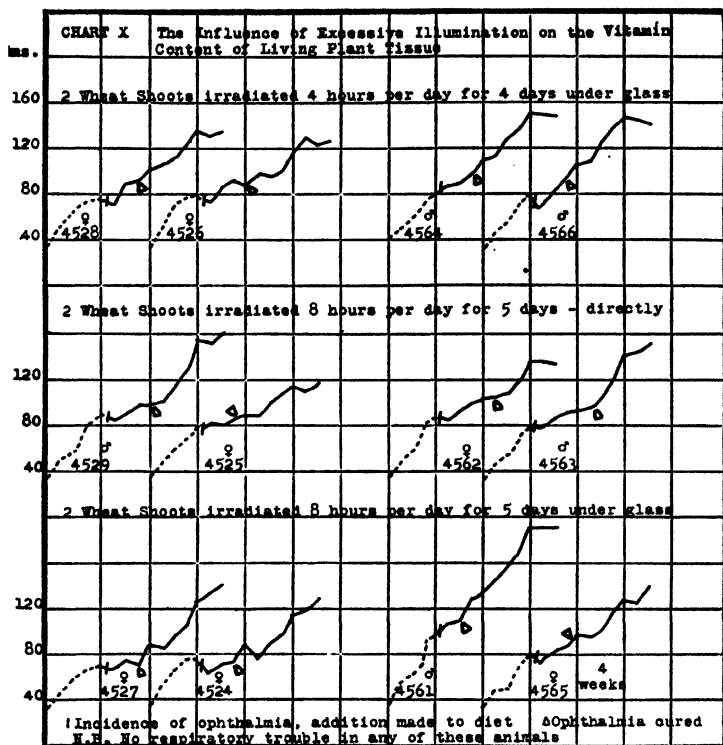


CHART X. Rats 4524 to 4529 were from one litter, Rats 4561 to 4566 from another litter. Rats 4528, 4526, 4564, 4566 receiving two wheat shoots irradiated 4 hours per day for 4 days under glass were used as controls. The other rats receiving two wheat shoots irradiated for 8 hours per day for 5 days whether directly or under glass showed that this excessive illumination had apparently had no deleterious effect on the vitamin content of the living shoots.

course, there are compensating accelerations or retardations in processes using up the vitamins.

In the foregoing experiments, it is remarkable to what extent the response given by the members of one litter is similar to that

given by the members of another. Different samples of apparently similar material have been used in many different experi-

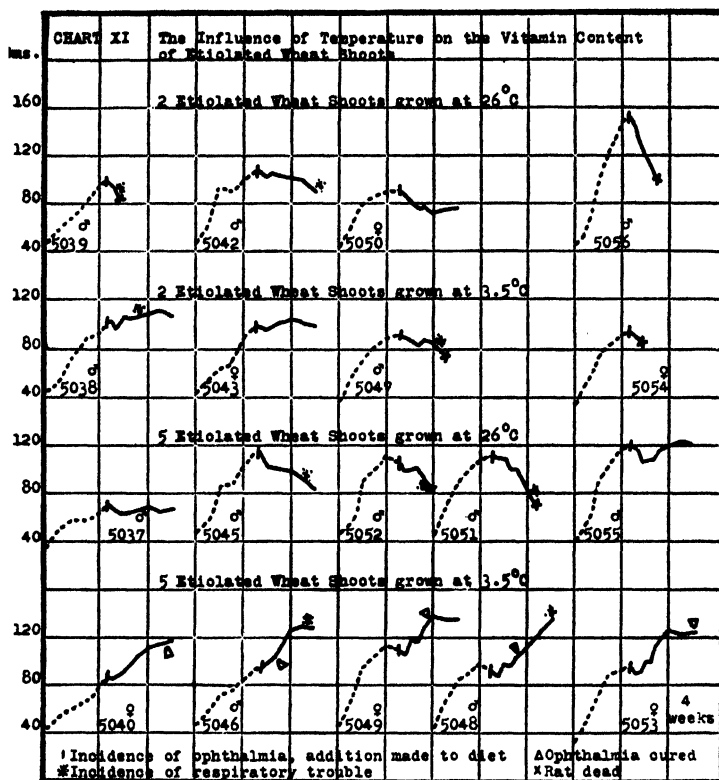


CHART XI. Rats 5037 to 5040 were from one litter, Rats 5042 to 5046 from another, Rats 5047 to 5052 from another, and Rats 5053 to 5056 from still another litter. Rats 5039, 5042, 5050, 5056 receiving two wheat shoots grown at 26°C. and Rats 5038, 5043, 5047, 5054 receiving two shoots grown at 35°C. showed very little evidence of the presence of vitamin A in the shoots.

Rats 5040, 5046, 5049, 5048, 5053 receiving five wheat shoots grown at the lower temperature, 3.5°C., showed that they contained distinctly more vitamin A than the shoots grown at the higher temperature, 26°C., and fed to Rats 5037, 5045, 5052, 5051, 5055.

ments for control or other tests and the only instance where results were not comparable appeared in the case of the etiolated wheat

shoots. Sometimes five of these shoots sufficed to maintain body weight and sometimes this dosage was enough to cure ophthalmia and also to make the animal resume growth for at least a short time. At first it seemed possible that, in spite of precautions taken to eliminate such variations in the rats, this might be due to individual differences only apparent when the dose of vitamin A was very small; but the remarkable uniformity in response observed when the other materials were tested, made it appear that some other factor had influenced the vitamin content of the etiolated shoots. The wheat seedlings, unlike the other plants, had at first been grown in an attic during October, November, and December, and in order to have shoots 10 to 12 cm. long, they had been allowed to grow for 16 days. The temperature of the attic as taken by a thermostat in early October for 2 weeks varied from 21-13°C. Later in the season the temperature gradually fell until the shoots had to be left to grow for about 24 days in order to attain the required size. In all the experiments in which this material was used, definite growth was obtained with five shoots. When, however, the temperature of the attic fell still further during late December and it was feared that the wheat would not grow at all, the flower pots were transferred to a dark room in the basement where the temperature was 26°C. and never varied more than 2°. Here it was found that the seedlings attained the desired weight in 7 days but the dosage of five shoots was only sufficient to cure ophthalmia and to promote growth for a very short time after which the animals lost weight and died (Chart XI).

The above consideration made it desirable to make a direct test for comparison between seedlings grown slowly at a low temperature and quickly at a high one. A cold potato storage cellar was used for the former, in which the temperature during the months of February, March, and April never fell below 3.5°C. or rose above 4.5°C. The seeds were allowed to germinate in pots in the warmer room for 5 days, *i.e.*, until their shoots were nearly 2 cm. long, and were then transferred, well covered, to the cold potato cellar. Growth was remarkably slow under the lower temperature and the oldest plants of the series had been in the cold cellar for 35 days before the shoots were equal in length to those grown for 7 days in the warmer room. The test on this

material was carried out at two levels (a) two shoots, and (b) five shoots. The test on the two shoot level showed practically no difference but at the five shoot level, the difference was remarkable. Shoots grown at the higher temperature did not cure ophthalmia nor did they always even maintain body weight, but those grown at the lower temperature cured ophthalmia and, for a time at least, caused resumption of growth (Chart XI).

#### SUMMARY.

Etiolated shoots of wheat and white and yellow corn may contain small quantities of vitamin A when grown to a height of about 10 to 12 cm. above the ground. Seedlings of wheat which have germinated for 2 days and have only made three rootlets and no shoot or only a very short one, do not contain any more vitamin A than similar ungerminated seeds.

The light from a quartz mercury vapor lamp is effective in accelerating the formation of vitamin A in living plant tissues.

The short ultra-violet rays from such a lamp, used in conjunction with the visible rays, do not have any influence on the ultimate amounts of vitamin A contained in the tissues.

Within reasonable limits, excessive illumination of the living plant tissue does not seem to have any deleterious effect on the vitamin A formed in it.

The amount of vitamin A in etiolated shoots is an inverse function of the temperature at which they have been grown, this having influenced markedly the rate of growth.

The writer desires to express her thanks to Professor H. Steenbock for his advice and her appreciation of the privilege of working at the University of Wisconsin and to the Rockefeller Foundation for a Medical Fellowship which made possible this year's work in the United States.

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## ON THE OXIDATION OF GLUCOSE IN ALKALINE SOLUTIONS OF IODINE.

BY WALTHER F. GOEBEL.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

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In 1918 Willstätter and Schudel (1) described a method for the estimation of sugars which embodied the principle first set forth by Romijn (2), that hexo-aldoses may be quantitatively oxidized to the corresponding hexonic acid in the presence of iodine in alkaline solution. The reaction may be expressed by the equation  $RCHO + I_2 + 3NaOH = RCOONa + 2NaI + 2H_2O$ . Thus 1 molecule of aldose reacts with 2 molecules of sodium hypoiodite, and when a measured excess of the latter is used the amount of iodine liberated on acidification, after the reaction between aldose and hypoiodite has reached completion, serves as a measure of the quantity of sugar present.

In the original description of their method Willstätter and Schudel state that sodium hydroxide must be slowly added to the sugar-iodine solution. It was observed by the author that if sodium hydroxide be added immediately to the glucose-iodine solution, or even over an interval of 30 to 60 seconds, one does not obtain quantitative oxidation of glucose. The success of the method depends therefore primarily on the rate of addition of sodium hydroxide to the mixture of glucose-iodine. These observations are summarized in Table I.

It is clearly seen that the rate of addition of alkali has a distinct influence on the success of the reaction glucose  $\rightarrow$  gluconic acid in reaching completion. If solutions of glucose-iodine, to which alkali has immediately been added, as in Experiments 6 to 8, are permitted to stand longer than 15 minutes, the oxidation of the sugar fails to come to completion, and at the end of a prolonged period of time, an hour or more, no hypoiodite can be demon-

strated in the reaction mixture. Apparently the oxidizing reaction has reached completion with some 4 per cent of glucose still unchanged. In the preparative method found in the following paper, this effect is shown to be greatly magnified. It is the nature of this phenomenon which will be discussed.

TABLE I.

*Effect of Rate of Addition of 2 Equivalents of 0.1 N Sodium Hydroxide on Oxidation of 10 Cc. of 0.9 Per Cent Glucose and 20 Cc. of 0.1 N Iodine-Potassium Iodide Solutions at 23°.*

Experiment No.	Glucose.	Time over which 2 equivalents of 0.1 N NaOH were added.	0.1 N iodine utilized in oxida- tion of glucose.	Glucose oxidized as calculated from iodine con- sumption.
	mg.	sec.	cc.	per cent
1	90	240	10.00	100
2	90	120	10.00	100
3	90	60	9.94	99.4
4	90	30	9.87	98.7
5	90	15	9.78	97.8
6	90	Immediately.	9.65	96.5
7	90	"	9.64	96.4
8	90	"	9.65	96.5

The method of analysis in Table I was carried out as follows: To 10 cc. of a 0.9 per cent glucose solution were added 20 cc. of 0.1 N iodine-potassium iodide solution and to this were added 2 equivalents of 0.1 N sodium hydroxide (40 cc. + 5 cc. to neutralize the gluconic acid formed). When the alkali was added over an interval, it was run from a burette at constant rate. When added "immediately" the required amount was quickly poured from a cylinder into the well stirred glucose-iodine solution. The whole operation required about 3 seconds. After standing a total of 15 minutes, when the reaction had come to completion, the mixture was acidified with 6 cc. of 1.0 N hydrochloric acid and was titrated with 0.05 N sodium thio-sulfate, using starch as an indicator. The method is accurate to about  $\pm 0.15$  per cent. The temperature variation was not greater than  $\pm 0.25^\circ$ . The sodium hydroxide used in these and subsequent experiments was exactly 0.1 N and was prepared from carbonate-free sodium hydroxide from sodium. The iodine-potassium iodide solution contained 25.0 gm. of the potassium iodide per liter.

Until the appearance of Schönbein's (3) "Beiträge zur nähern Kenntniss des Sauerstoffs und der einfachen Salzbildner" it was believed that iodine and potassium hydroxide in solution reacted instantaneously to form potassium iodate. Schönbein showed this

reaction to take place in two stages, and from the analogy between the chemical similarity of iodine and chlorine, assumed that an intermediate product, potassium hypoiodite, was formed. Schönbein's experiments showed that the first stage of the reaction, the formation of hypoiodite, is incomplete and reversible, and that an equilibrium between alkali, iodine, iodide, and hypoiodite existed. With regard to the second reaction, the formation of iodate from hypoiodite, he says little except that the velocity is increased as the temperature is raised. This second reaction has been studied in great detail by many investigators and an excellent review of the literature is to be found in the articles of Skrabal (4) which appeared in 1907. Most of these studies have been carried out in solutions of high dilution, but Taylor (5) has demonstrated that in solutions of decinormal strength the formation of iodate from hypoiodite progresses at a remarkable rate.

In an attempt to obtain a more accurate knowledge of the rate of formation of iodate from hypoiodite in solutions of the same concentration as those employed in the Willstätter-Schudel method, a short series of experiments was carried out and the results are given in Table II. A second series of experiments was also carried out concerning the effect of the rate of addition of alkali on the rate of oxidation of glucose and on the rate of the formation of iodate from hypoiodite in these sugar-containing solutions. These results are found in Table III.

From the data presented in these tables there are two facts worthy of note: first, in the absence of glucose the rate of addition of 0.1 N sodium hydroxide to 0.1 N iodine solution has no influence on the speed of formation of iodate from hypoiodite over the time intervals studied; and second, the rate of addition of alkali has a marked influence on the speed of formation of iodate from hypoiodite in a solution containing glucose. It is to be borne in mind that in an alkaline glucose-iodine solution in addition to the reaction (1)  $\text{RCHO} + 3\text{NaOH} + \text{I}_2 = \text{RCOONa} + 2\text{NaI} + 2\text{H}_2\text{O}$  there is going on simultaneously a second and equally important reaction (2)  $3\text{NaOI} = \text{NaIO}_3 + 2\text{NaI}$ . In order to explain the phenomenon of the failure of glucose to be oxidized quantitatively when alkali is immediately added to glucose-iodine solution, it becomes necessary to take into consideration the reaction velocity of this second reaction.

Before going on with this discussion, however, an important factor which determines the success of reaction (1) must be pointed out. This factor is the necessity for the presence, as the

TABLE II.

*Rate of Formation of Iodate from Hypiodite in Solution of 20 Cc. of 0.1 N Iodine, 10 Cc. of Water, and 40 Cc. of Sodium Hydroxide at 23°.*

Series No.	Time of reaction.	Hypiodite-iodide in solution.	Iodate-iodide in solution.
	min.	per cent	per cent
I	2	17.8	82.0
	7	5.8	94.2
	12	3.7	96.1
II	2	18.0	82.0
	7	4.5	95.6
	12	3.7	96.2

The method of procedure in Table II was as follows: In Series I 2 equivalents (40 cc.) of 0.1 N sodium hydroxide were immediately added to 20 cc. of 0.1 N iodine-potassium iodide solution and 10 cc. of water. The mixture was permitted to stand the required length of time, then to it were added 2 gm. of solid sodium bicarbonate and 100 cc. of water. A blast of carbon dioxide was passed through to neutralize the free alkali. In this manner the iodine from the hypiodite and unaltered iodine was liberated. 15 cc. of 0.1 N sodium arsenite were added and the excess arsenite was titrated with 0.1 N iodine-potassium iodide solution, using starch as indicator. The back titration with 0.1 N iodine in cc. minus 15 cc. is equal to the cc. of iodine in the form of hypiodite and iodine in equilibrium. The iodate was then determined by adding small portions of HCl to this solution and titrating with 0.05 N sodium thiosulfate. In Series II 2 equivalents of 0.1 N alkali were added at constant rate from a burette over an interval of 2 minutes, and the analyses made exactly as above. The time of reaction was counted from the first addition of sodium hydroxide. The temperature variation, as in all these experiments was not greater than  $\pm 0.25^\circ$ . Since iodine in sodium hydroxide exists in equilibrium form,  $2I + NaOH \rightleftharpoons NaOI + HI$ , and  $3NaOI = NaIO_3 + 2NaI$ , the so called "hypiodite" determination represents all of the iodine in the first equation; the "iodate" determination represents the right hand member of the second equation.

reaction approaches completion, of a concentration of hypiodite sufficient to drive it to the end. If the concentration falls below a certain optimum, reaction (1) will never reach completion. The condition which determines this factor is the rate of addition of sodium hydroxide.

A sufficiently high concentration of hypiodite is possibly not attained when alkali is added immediately to a glucose-iodine

TABLE III.

*Influence of Rate of Addition of 2 Equivalents of 0.1 N Sodium Hydroxide on Rate of Formation of Iodate from Hypiodite and Rate of Oxidation of Glucose in Alkaline Hypiodite Solution at 23°.*

Series No.	Glucose.	Time.	0.1 N iodine in form of:		0.1 N iodine consumed by glucose.	Iodine-hypiodite in solution.	Hypoiodite converted to iodate-iodide.	Glucose oxidized.
			Iodine-hypiodite.	Iodate-iodide.				
	mg.	min.	cc.	cc.	cc.	per cent	per cent	per cent
I	90	2	1.45	10.00	8.55	12.7	87.3	85.5
		5	0.48	10.16	9.36	4.5	95.5	93.6
		8	0.42	10.16	9.42	4.0	96.0	94.2
		11	0.22	10.26	9.52	2.1	97.9	95.2
		15	0.12	10.27	9.61	1.1	98.9	96.1
		30	0.05	10.27	9.68	0.5	99.5	96.8
		120	None.	10.35	9.65	0.0	100.0	96.5
		240	"	10.35	9.65	0.0	100.0	96.5
II	90	2	3.10	7.60	9.30	29.0	71.0	93.0
		5	1.57	8.61	9.82	15.5	84.5	98.2
		8	0.84	9.21	9.95	8.4	91.6	99.5
		11	0.65	9.36	9.99	6.5	93.5	99.9
		15	0.64	9.38	9.98	6.4	93.6	99.8
		30	0.30	9.69	10.01	3.0	97.0	100.1
		120	0.07	9.93	10.00	0.7	99.3	100.0
		240	0.03	9.97	10.00	0.3	99.7	100.0

The method of procedure in Table III was as follows: To 10 cc. of 0.9 per cent glucose and 20 cc. (2 equivalents) of 0.1 N iodine-potassium iodide solutions were added 2 equivalents (40 cc. + 5 cc. to neutralize the gluconic acid formed) of 0.1 N sodium hydroxide, either immediately, as in Series I, or over an interval of 2 minutes at constant rate from a burette, as in Series II. At the end of the time interval (tabulated in the third column) counted from the first addition of alkali, the analyses for "hypiodite" and "iodate" were made as under Table II. The iodine consumed in the oxidation of glucose represents the sum of the hypiodite-iodate titration subtracted from 20. The percentages of hypiodite and iodate are those calculated for iodine unconsumed by glucose.

solution for the reason that reactions (1) and (2) start out simultaneously. Under these conditions reaction (1), though it has a

greater *initial* speed than reaction (2), will be overtaken by the second as the two near completion with the result that at this point the concentration of hypiodite falls below the necessary optimum and both reactions end with the hypiodite completely converted to iodate and the glucose in an incomplete state of oxidation. If, on the other hand, the alkali be added over an interval of 2 minutes, the major part of the hypiodite will enter into the first reaction as it is formed, since the *initial* speed of the reaction glucose  $\rightarrow$  gluconic acid is greater than that of the reaction hypiodite  $\rightarrow$  iodate. In other words, during the addition of the first increments of alkali the concentration of hypiodite in the solution would be kept low, a condition unfavorable for the reaction hypiodite  $\rightarrow$  iodate.

A second interpretation might explain the phenomenon of the failure of quantitative oxidation when alkali is immediately added to glucose-iodine solutions; namely, the initial high concentration of hydroxyl ions would favor any process of enolization of glucose to unoxidizable ketose. If this be the case, then if glucose and alkali be first mixed and iodine be added to the mixture over an interval of 2 minutes, one should obtain 96 per cent oxidation. As a matter of fact, one obtains some 98 per cent oxidation. The discrepancy is even more striking if one permits glucose and 0.1 N alkali to stand 12 hours before adding iodine, for nearly 25 per cent of glucose will remain unaccounted for. It may be assumed therefore, that a small amount of glucose is actually converted to ketose under these conditions, and that the rapid conversion of hypiodite to iodate does not completely explain the phenomenon of incomplete oxidation.

If potassium iodide be added in excess to iodine-glucose solutions (thus tending to increase the concentration of hypiodite by repressing the reaction  $3\text{NaOI} = \text{NaIO}_3 + 2\text{NaI}$ ), and then 2 equivalents of sodium hydroxide be added at one time, the oxidation progresses approximately to the same point as when iodine is added over an interval of 2 minutes to glucose-alkali solutions. This observation confirms the assumption that a high initial hydroxyl ion concentration hinders in part the reaction glucose  $\rightarrow$  gluconic acid from going to completion despite a high hypiodite concentration at the end of the reaction. Whether this hindering effect can be attributed entirely to a process of enolization of the

sugar, to an anticatalytic effect, or to some other remote cause is of course difficult to say when one deals with highly complex alkaline glucose-iodine solutions in which rapid concentration changes are taking place.

#### SUMMARY.

An explanation, based on the necessity of a proper hydroxyl ion concentration, for the stoichiometrical progression of the reaction glucose  $\rightarrow$  gluconic acid in alkaline solutions of iodine, has been offered, and found to agree with the observed reaction curves.

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# THE PREPARATION OF HEXONIC AND BIONIC ACIDS BY OXIDATION OF ALDOSES WITH BARIUM HYPOIODITE.

By WALTHER F. GOEBEL.

(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)

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## INTRODUCTION.

In seeking a method for the conversion of an aldose to the corresponding monobasic sugar acid the chemist as a rule reverts to the classical principle used to such great advantage by Emil Fischer. As a reagent for bringing about this conversion bromine does not possess all the advantages which might be desired, for in some instances, in addition to the great period of time necessary for the oxidation to reach completion, it is difficult to control the conditions of reaction in a manner so as to yield an end-product free from the unchanged parent substance and from products of further oxidation.

During the course of an investigation (1) on the chemical nature of an aldobionic acid isolated as a hydrolytic product from the specific polysaccharide produced by Type III pneumococcus during growth, it became necessary to oxidize this aldehydic sugar acid as a step in the elucidation of its structure. For purposes of oxidation bromine failed. The present study found its impetus in this problem and has as its objective a general method having the advantages of good yields, freedom from too many technical difficulties, and finally in bringing about the conversion, aldose  $\rightarrow$  hexonic acid, unfailingly and quantitatively.

A review of the various methods employed in the quantitative estimation of reducing sugars reveals only one in which the reaction between aldose and oxidant progresses stoichiometrically. The reaction  $RCHO + 3NaOH + I_2 = RCOONa + 2NaI +$

$2\text{H}_2\text{O}$  was first employed in principle by Romijn (2), later by Colin and Liévin (3), and was finally shown to progress in a stoichiometric manner under the conditions prescribed in the ingenious method of Willstätter and Schudel (4). It was thought possible to make use of the above reaction in devising a method for preparing hexonic and bionic acids. There is however a serious disadvantage in using potassium iodide-iodine and sodium hydroxide to carry out this oxidation, for it is obviously most difficult to rid the reaction product of these inorganic constituents. There is a second disadvantage in following implicitly the directions of Willstätter and Schudel in regard to the concentration of reagents; namely, the necessity of employing an enormous fluid bulk for the oxidation of a few gm. of material. To eliminate these two difficulties it was thought possible to substitute barium iodide-iodine and barium hydroxide for the reagents of Willstätter and Schudel and to carry out the oxidation in solutions of higher concentration. If the conditions of reaction be properly regulated it should still progress in the manner  $2\text{RCHO} + 3\text{Ba}(\text{OH})_2 + 2\text{I}_2 = (\text{RCOO})_2\text{Ba} + 2\text{BaI}_2 + 4\text{H}_2\text{O}$  and one should ultimately obtain in the reaction mixture inorganic constituents which can be eliminated with the greatest ease, leaving in the mother liquid the desired sugar acid as end-product.

#### EXPERIMENTAL.

##### *Methods.*

In order to ascertain whether glucose could be quantitatively oxidized to gluconic acid analyses were made under the conditions described by Willstätter and Schudel but substituting 0.1 N barium hydroxide and 0.1 N iodine-barium iodide solutions for the usual reagents.

The method of procedure was as follows:

20 cc. of 0.1 N iodine-barium iodide solution (containing 25 gm. of barium iodide per liter) were added to 10 cc. of 0.9 per cent glucose solution and to the mixture were then added 45 cc. of 0.1 N barium hydroxide at constant rate from a burette. After standing for 15 minutes the solution was acidified with a slight excess of normal hydrochloric acid and the liberated iodine was titrated with 0.05 N sodium thiosulfate, using starch as indicator.

The results which are given in Table I show that barium hypoio-

dite is just as effective as is sodium hypoiodite in bringing about a quantitative oxidation of glucose to gluconic acid.

A second study was undertaken to ascertain whether glucose could be oxidized quantitatively in solutions of barium hypoiodite of higher concentration than decinormal, and whether the addition rate of barium hydroxide, as well as its final concentrations, would affect the ultimate outcome of the stoichiometry of the oxidation. Barium hydroxide of 0.4 N (approximately a saturated solution at room temperature) and 0.3 N iodine were chosen for this study. The analyses were carried out in the following manner.

90 mg. of solid crystalline glucose were added to 7 cc. of 0.3 N iodine-barium iodide (containing 75 gm. of barium iodide per liter). To this solution was then added the number of equivalents of barium hydroxide as indicated

TABLE I.

*Relative Oxidizing Power of 0.1 N Iodine-Potassium Iodide and 0.1 N Iodine-Barium Iodide on Glucose in Alkaline Solutions.*

Series I. Using 0.1 N potassium iodide-iodine and 2 equivalents of 0.1 N sodium hydroxide solutions.

Series II. Using 0.1 N barium iodide-iodine and 2 equivalents of 0.1 N barium hydroxide solutions.

Glucose.	0.1 N iodine utilized.	Glucose oxidized.	Glucose.	0.1 N iodine utilized.	Glucose oxidized.
mg.	cc.	per cent	mg.	cc.	per cent
45	4.98	99.6	45	4.98	99.6
90	10.00	100.0	90	9.99	99.9
135	14.98	99.9	135	14.97	99.8

in the third column of Table II, plus 1.25 cc. to neutralize the gluconic acid formed, from a burette at constant rate over the interval of time indicated in the fourth column. The mixture was permitted to stand 15 minutes and was then titrated, after acidification with a slight excess of normal hydrochloric acid, with 0.05 N sodium thiosulfate solution, using starch as indicator. The barium hydroxide used in these experiments was prepared by recrystallizing Merck's reagent barium hydrate 8 times from water. A solution of this, when exactly neutralized with sulfuric acid and freed from barium sulfate, gave no visible residue on evaporation.

Sulfuric acid may not be used for acidification because not all of the iodine originally present can be accounted for. This disappearance of iodine is not entirely a phenomenon of adsorption for it was observed that the longer the solutions stood before acidification the greater was the loss of iodine. Since the formation of barium iodate takes place quickly in the solutions, this loss of iodine may be due to the formation of an insoluble barium-iodate-sulfate complex.

The results, as given in Table II, demonstrate that glucose is more than quantitatively oxidized to gluconic acid as measured by the iodine consumption, and that the base must be added to the iodine-glucose solution over an interval of time greater than 2 minutes in order to achieve such oxidation. It is seen, furthermore, that for oxidation to reach the end-point it is unnecessary to have more than 1 equivalent of base in the solution.

Although glucose is in part oxidized beyond the gluconic acid stage it was thought wise to choose the higher concentrations of barium hydroxide and iodine-barium iodide as reagents for pre-

TABLE II.

*Effect of Rate of Addition and Ultimate Concentrations of 0.4 N Barium Hydroxide on Oxidation of Crystalline Glucose Dissolved in 0.3 N Iodine-Barium Iodide Solution at 23°.*

Glucose.	0.4 N Ba(OH) <sub>2</sub> added.	Equivalents of Ba(OH) <sub>2</sub> .	Time over which Ba(OH) <sub>2</sub> was added.	0.3 N iodine utilized by glucose.	Glucose.
mg.	cc.		min.	cc.	per cent
90	6.25	1.00	3	3.55	106.5
90	7.81	1.25	3	3.54	106.2
90	9.10	1.50	3	3.55	106.5
90	11.25	2.0	Immediately.	2.00	66.0
90	11.25	2.0	2	3.15	94.5
90	11.25	2.0	3	3.54	106.2
90	11.25	2.0	4	3.56	106.8
90	38.75	6.0	4*	3.52	105.6

\* First 2 equivalents were added over 3 minutes and the remaining 4 equivalents over 1 minute.

paring sugar acids on a large scale, rather than decinormal solutions, because it was felt that the great economy of fluid bulk would offset the loss of end-product through processes of purification. Glucose, maltose, and lactose were subjected to oxidation, and the corresponding gluconic, maltobionic, and lactobionic acids were isolated as described below.

#### *Preparation of Calcium Gluconate.*

9.0 gm. of crystalline glucose were dissolved in 666 cc. of 0.3 N barium iodide-iodine<sup>1</sup> solution and to it while stirring was added 1 liter of 0.4 N

<sup>1</sup> Prepared by dissolving 75 gm. of Merck's barium iodide and 38.1 gm. of resublimed iodine in water and diluting to 1 liter.

barium hydroxide at constant rate of flow over an interval of 3 minutes. The mixture was allowed to stand 15 minutes and was then acidified with 18.5 cc. of concentrated sulfuric acid dissolved in 150 cc. of water, after which an excess (150 gm.) of lead carbonate (Kahlbaum I) was immediately added. The mixture after rapid mechanical stirring, became neutral to Congo red paper. When this point was reached the precipitate was permitted to settle and the supernatant liquid, which contained lead gluconate, was evaporated *in vacuo*. The precipitate of lead carbonate, lead iodide, and barium sulfate was centrifuged and washed several times and the washings were added to the above supernatant liquid. The distillation *in vacuo* rids the solution of iodine. The concentrated aqueous solution of lead gluconate was filtered, the lead was precipitated with a slight excess of sulfuric acid, and the small traces of hydriodic acid still in solution were removed by the addition of a small amount of silver sulfate. After filtration of the silver iodide, the remaining silver and traces of lead in the filtrate were removed with hydrogen sulfide. One thus obtains a solution which should contain no inorganic constituent save sulfate ion which may be quantitatively removed by barium hydroxide. The final filtrate, which contained free gluconic acid, was boiled with an excess of calcium carbonate and a small amount of norit, and after cooling was filtered. The resulting solution of calcium gluconate was quite water-clear and colorless. It was evaporated *in vacuo* to a syrup and was poured into 10 volumes of redistilled methyl alcohol. 10.8 gm., or 91 per cent of the theory, of crude calcium gluconate were recovered. When recrystallized from water 8.2 gm. of crystalline calcium gluconate were obtained.

0.1002 gm. substance: 0.1234 gm.  $\text{CO}_2$  and 0.0467 gm.  $\text{H}_2\text{O}$ .

0.1688 " " : 0.0219 "  $\text{CaO}$ .

	per cent	per cent	per cent
Calculated for $(\text{C}_6\text{H}_{11}\text{O}_7)_2\text{Ca}$ .	C 33.47,	H 5.15,	Ca 9.31.
Found.	" 33.59,	" 5.21,	" 9.27.

#### *Preparation of Calcium Maltobionate.*

This product was prepared from 18 gm. of maltose exactly as was calcium gluconate. The crude product, which is also obtained in yields which vary between 80 and 90 per cent, always analyzes high in carbon and hydrogen, and low in calcium. In order to obtain a product which analyzes correctly the crude material is purified according to the method of Levene (5). This consists in dissolving the salt in approximately 3 times its weight of water, and precipitating in a centrifuge bottle with  $1\frac{1}{2}$  volumes of alcohol. About 50 to 60 per cent of salt is thus precipitated. The mixture is centrifuged and the supernatant liquid is poured off and saved for reworking, while the only calcium maltobionate is dissolved in a small amount of water and precipitated by throwing into 10 to 15 volumes of methyl alcohol. Two different preparations were made in this manner.

Preparation 1. 0.0906 gm. substance: 0.1272 gm.  $\text{CO}_2$  and 0.0456 gm.  $\text{H}_2\text{O}$ .

0.1784 gm. substance: 0.0130 gm.  $\text{CaO}$

		per cent	per cent	per cent
Calculated for $(C_{12}H_{21}O_{13})_2Ca$ .	C	38.18,	H 5.6,	Ca 5.31.
Found.	"	38.28,	" 5.63,	" 5.21.
Preparation 2.	0.1180 gm. substance:	0.1676 gm. $CO_2$	and 0.0600 gm. $H_2O$ .	
	0.1153 gm. substance:	0.0090 gm. $CaO$ .		
		per cent	per cent	per cent
Found.	C	38.73,	H 5.68,	Ca 5.55.

*Preparation of Calcium Lactobionate.*

This product was prepared and purified as was calcium maltobionate.  
 0.1178 gm. substance: 0.1663 gm.  $CO_2$  and 0.0600 gm.  $H_2O$ .

0.1208 gm. substance: 0.0089 gm.  $CaO$ .

Found. C 38.50, H 5.73, Ca 5.30.

All analytical samples were dried to constant weight *in vacuo* over successive baths of carbon tetrachloride, water, and toluene.

Dr. P. A. Levene was kind enough to suggest the method of purification of crude calcium maltobionate and lactobionate; without his suggestion this method would not have proven feasible.

It might be suggested that this method may be used in the preparation of acids of the methylated sugars, since Sobotka (6) has shown that they react stoichiometrically with sodium hypoiodite. In cases in which only 1 or 2 gm. of valuable material are to be subjected to oxidation by this method it is advisable to use solutions of decinormal strength.

## CONCLUSIONS.

A method for the preparation of hexonic and bionic acids which makes use of barium hypoiodite as an oxidant has been described. This method possesses the advantage of speed, of ease in manipulation, of furnishing good yields, and of employing inorganic reagents which may ultimately be eliminated quickly and quantitatively, leaving in solution an end-product readily purified.

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## THE EFFECT OF IONIZATION UPON OPTICAL ROTATION.

### II. RELATIONS IN THE SERIES OF AMINO ACIDS, POLYPEPTIDES, AND KETOPIPERAZINES.

By P. A. LEVENE, LAWRENCE W. BASS, ROBERT E. STEIGER, AND ISAAC BENCOWITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

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It has long been known that for any given optically active electrolyte the ion and the undissociated molecule generally exhibit individual and characteristic optical rotations. The change in rotation due to a change in the equilibrium between ions and undissociated molecules can therefore be employed in measuring the degree of dissociation of an optically active electrolyte as a function of external conditions, such as hydrogen ion concentration. The method can likewise be applied to the determination of dissociation constants and it should prove to be of particular interest in the case of dissociation constants which lie in ranges in which the electrometric method no longer permits accurate measurement.

It has recently been shown by Levene, Simms, and Bass<sup>1</sup> for nucleotides that a plot of molecular rotation  $[M]$  against corrected equivalents of base  $b'$  gives straight line curves between the integral values of  $b'$ . Also Wood,<sup>2</sup> Vlès and Vellinger,<sup>3</sup> and Vellinger<sup>4</sup> have investigated the relation between optical rotation and ionization.

In the present paper the method has been applied to a study of enolization in peptides and ketopiperazines.

<sup>1</sup> Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 1926, lxx, 243.

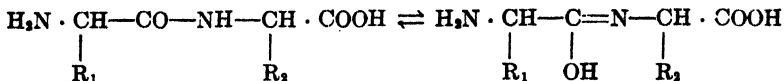
<sup>2</sup> Wood, J. K., *J. Chem. Soc.*, 1914, cv, 1988.

<sup>3</sup> Vlès, F., and Vellinger, E., *Compt. rend. Acad.*, 1925, clxxx, 742.

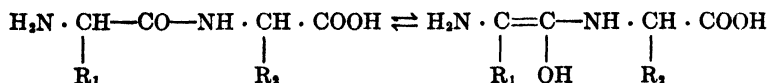
<sup>4</sup> Vellinger, E., *Compt. rend. Acad.*, 1926, clxxxii, 1625.



The phenomenon of enolization in proteins has been assumed by many authors. This enolization was generally thought to occur in the imide group:



Dakin<sup>5</sup> was the first, to our knowledge, to suggest still another form of enolization involving 2 carbon atoms:



According to Dakin, this enolization takes place in peptides only when the chain contains at least three amino acids. Levene and Pfaltz<sup>6</sup> have recently shown that enolization of this type takes place more readily in ketopiperazines and to a less degree in polypeptides. Thus it is possible that in polypeptides the first type of enolization predominates, whereas in ketopiperazines the second type prevails. Enolization of either type, however, leads to the formation of an additional ionizable group of which the degree of dissociation should be measurable by the polarimetric method.

A study of dextro-alanyl-dextro-alanine anhydride was made first, in view of the fact that this substance contains no free carboxyl or amino groups; hence, any buffer effect produced by it may justly be attributed to enolization. Indeed, E. Fischer, having observed the ready hydrolysis of ketopiperazines by alkali, assumed an intermediate phase of salt formation due to enolization. Euler and Pettersson<sup>7</sup> have recently expressed a similar view and they estimated a value for the dissociation constant of glycyl-glycine anhydride of the order of magnitude of  $10^{-15}$ . By means of the polariscopic method we have determined the dissociation constant of dextro-alanyl-dextro-alanine anhydride with an approximate value of  $10^{-13.5}$  ( $\text{pG}' = 13.5 \pm 0.1$ ).

<sup>5</sup> Dakin, H. D., *J. Biol. Chem.*, 1912-13, xiii, 357.

<sup>6</sup> Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183; *J. Biol. Chem.*, 1926, lxxviii, 277; 1926, lxx, 219.

<sup>7</sup> Euler, H., and Pettersson, E., *Z. physiol. Chem.*, 1926, clviii, 7.

The method was then applied to a tetrapeptide, glycyl-levo-alanyl-levo-alanyl-glycine. This substance showed a considerable change in rotation when the hydrogen ion concentration was reduced to pH 13.35 by the addition of 5 mols of alkali. It is unfortunate that lack of material prevented us from making more than one measurement in the third buffer range. However, the fact that the two dissociation constants (due to the carboxyl and amino groups) of the tetrapeptide are of the same order of magnitude as those previously determined for amino acids and simpler peptides warrants the conclusion that the third change in rotation is due to one or more additional dissociation constants, namely the enolic dissociation constants.

In order to test whether this change might have been due to the effect of a high concentration of alkali, the titration-rotation data were determined for dextro-alanine. In this case no abnormality was observed in the data; *i.e.*, no further changes in rotation were observed when the pH was raised far beyond the range of the second dissociation constant.

From the data given in the experimental part it is seen that the agreement between the electrometric and polarimetric values of the degree of dissociation is such as may be expected from the precision of our polarimeter, which is accurate to  $\pm 0.02^\circ$ . With an apparatus of greater precision we hope to obtain very accurate values of ionic concentrations.

#### EXPERIMENTAL.

##### 1. Preparation of Compounds.

*Dextro-alanine* was obtained from the hydrolytic products of silk by the procedure described by Fischer.<sup>8</sup>

*Dextro-alanyl-dextro-alanine anhydride* was prepared from dry dextro-alanine ethyl ester.<sup>9</sup> 46.9 gm. were heated 24 hours in an oil bath at 100°C. The crystals of anhydride which separated were filtered off by suction and were then washed repeatedly with ether. The yield was 5 gm. (17 per cent of the theory). The product was purified by recrystallization from 10 times its weight of boiling water.

<sup>8</sup> Fischer, E., *Ber. chem. Ges.*, 1906, xxxix, 462.

<sup>9</sup> Cf. Fischer, E., *Ber. chem. Ges.*, 1906, xxxix, 468.

*Glycyl-levo-alanyl-levo-alanyl-glycine* was prepared according to the directions of Levene and Pfaltz.<sup>10</sup>

### 2. Polarimetric and Potentiometric Measurements.

*Preparation of Solutions.*—All solutions were prepared from analyzed samples and the respective concentrations were corrected on the basis of the analyses.

*Measurement of Rotations.*—The rotations were measured at 25°C. for the wave-length  $\lambda = 5461 \text{ \AA}$ , obtained from the light of a mercury arc by purifying it with a direct-vision spectroscope, in 2.00 dm. open tubes (with ground glass covers) containing 4 cc., or in 4.00 dm. open tubes (with glass plate covers) containing 22 cc.

The values recorded in the tables represent the mean of a series of at least six readings. The accuracy of the polarimeter was  $\pm 0.02^\circ$ . The relative values, however, were much better.

*Measurement of pH Values.*—The pH measurements were made at 25°C. in water-jacketed hydrogen electrode cells. The pH value 1.075 of 0.1000 N HCl was used as a standard. The saturated KCl liquid junction was assumed constant.

### 3. Calculations.

*Titration Data.*—The calculations from the titration data were made by the method described previously,<sup>11</sup> the following equations being employed:<sup>12</sup>

$$ph = pH - \log \tau_H \quad (1)$$

$$poh = (13.89 - pH) - \log \tau_{OH} \quad (2)$$

$$b' = \frac{b - a}{\gamma} + \frac{h - oh}{\gamma} \quad (3)$$

<sup>10</sup> Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1926, lxx, 219.

<sup>11</sup> Levene, P. A., Bass, L. W., and Simms, H. S., *J. Biol. Chem.*, 1926, lxx, 229. Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

<sup>12</sup> The notation is the same as that employed in previous papers except that  $\gamma$  is used for degree of dissociation instead of  $\alpha$  to avoid confusion with  $\alpha$  denoting rotation.

where  $H$  refers to activities and  $h$  and  $oh$  to concentrations. The values of  $pG'$  were obtained from the relation

$$pG' = pH - \log \frac{\gamma}{1 - \gamma} \quad (4)$$

*Rotation Data.*—The theory of the calculations from the rotation data is discussed in a previous paper.<sup>13</sup>

The degree of dissociation was calculated from the equation

$$\gamma = \frac{[M] - [M_u]}{[M_m] - [M_u]} \quad (5)$$

in which  $[M_u]$  is the rotation of the undissociated substance,  $[M_m]$  the rotation of the monion, and  $[M]$  the observed rotation. From the values of  $\gamma$  (or directly from the rotation values) the dissociation constant was calculated by means of the relation.

$$pG' = pH - \log \frac{\gamma}{1 - \gamma} = pH - \log \frac{[M] - [M_u]}{[M_m] - [M_u]} \quad (6)$$

It should be noted that from equation (5) either  $[M_u]$  or  $[M_m]$  can be calculated when  $[M_m]$  or  $[M_u]$  and a value of  $\gamma$  with the corresponding  $[M]$  are known:

$$[M_m] = \frac{[M_u] (\gamma - 1) + [M]}{\gamma} \quad (7)$$

*Dextro-Alanine and Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.*—For these ampholytes the two dissociation constants were calculated without making any assumptions in regard to the basic or acidic character of the two ionizing groups. The rotation of the molecular species existing in aqueous solution is designated as  $[M_0]$ , that on the acid side as  $[M_1]$ , and that on the alkaline side as  $[M_2]$ . Hence

$$\gamma_1 = \frac{[M] - [M_1]}{[M_0] - [M_1]} \quad (8)$$

$$\gamma_2 = \frac{[M] - [M_0]}{[M_2] - [M_0]} \quad (9)$$

<sup>13</sup> Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 1926, lxx, 243.

*Dextro-Alanyl-Dextro-Alanine Anhydride*.—Preliminary experiments showed that this compound was hydrolyzed quite rapidly under the conditions of our experiments. It was therefore necessary to correct the data for hydrolysis, the correction being made as follows.

Levene and Pfaltz<sup>14</sup> showed that dextro-alanyl-dextro-alanine anhydride was rapidly hydrolyzed by a large excess of alkali and that under these conditions no perceptible racemization occurred.

A sample of the anhydride with 15 equivalents of alkali was allowed to stand at room temperature for 2 hours. Rotations of this hydrolyzed material were then taken for solutions containing 0, 1.000, 3.00, 5.00, and 15.00 equivalents of alkali, respectively. On the basis of the results (Table II)  $\alpha_h = -0.19^\circ$  was taken as the rotation in a 4.00 dm. tube of a 0.0200 M solution of the hydrolyzed anhydride throughout the alkaline range.

The titration-rotation data were then determined. After each experiment samples of the solution were neutralized exactly and diluted to 0.0200 M. From the rotations of these solutions the degree of hydrolysis ( $h$ ) was calculated by means of the relation

$$h = \frac{\alpha_0 - \alpha}{\alpha_0 - \alpha_h}, \quad (10)$$

where  $\alpha_0$  is the rotation of the anhydride in neutral solution (no hydrolysis),  $\alpha_h$  the rotation of the hydrolyzed material (dipeptide), and  $\alpha$  the rotation of the neutralized solution. Because of the low rotations the values of  $h$  (Column 7, Table III) were only approximate, but they were sufficiently accurate for the succeeding calculations.

The degree of dissociation of the enolized anhydride in each of the original solutions was then calculated by the following method. In each solution the observed rotation, calculated as molecular rotation  $[M_x]$ , is the sum of two rotations:

$$[M_x] = h[M_h] + (1 - h)[M], \quad (11)$$

where  $h$  is the per cent of hydrolysis,  $[M_h]$  the molecular rotation of the hydrolyzed anhydride, and  $[M]$  the molecular rotation of the unhydrolyzed fraction (equilibrium mixture of the ionized

<sup>14</sup> Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxxiii, 661.

and unionized substance). The true values for the rotations of the anhydride ( $[M]$ ) were obtained from this equation (Column 8, Table III).

Since it was not possible to determine directly  $[M_m]$ , the rotation of the completely ionized enolic form, its value was obtained by extrapolation. The  $b'$  values, which are equal to  $\gamma$ , for 0.500, 0.800, and 1.000 equivalents of alkali were calculated from the titration data<sup>15</sup> (Column 3, Table III). The values for  $[M_m]$  calculated by equation (7) for these points were +72.5, +70.5, and +63.5, respectively. Using the average value +68.5 for  $[M_m]$ , -46.5 for  $[M_u]$ , and the rotations  $[M]$  of the anhydride at different degrees of dissociation, the values of  $\gamma$  (Column 9, Table III) were calculated from equation (5). The  $pG'$  values (Column 10, Table III) were calculated from equation (4).

#### 4. Experimental Data.

*Dextro-Alanine.*—The dextro-alanine used in these experiments gave the following analysis.

No. 547.

Calculated for  $C_3H_7O_2N$ . C 40.42, H 7.92, N 15.73.

Found. " 40.36, " 7.81, " 15.95 (Kjeldahl).

Individual samples of alanine equivalent to a 0.750 M solution were weighed into flasks. The solutions were then made up to 25.0 cc. with the required volumes of standard acid (or alkali) and water. The rotations were measured in 4.00 dm. tubes.

The data are recorded in Table I. The rotation values used for the calculation of  $\gamma_1$  and  $\gamma_2$  are  $[M_1] = 15.18$ ,  $[M_0] = 2.13$ , and  $[M_2] = 4.80$ . Column 14 gives the deviations of  $pG'$  calculated on the basis of rotation from the average values determined by titration.

*Dextro-Alanyl-Dextro-Alanine Anhydride.*—The material used in these experiments gave the following analysis.

No. 656.

Calculated for  $C_6H_{10}O_2N_2$ . C 50.69, H 7.09, N 19.72.

Found. " 50.32, " 7.26, " 19.79 (Kjeldahl).

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<sup>15</sup> The values of  $\gamma$  for higher concentrations of alkali were not reliable because of the hydrolysis which occurred.

TABLE I.  
*Dezdro-Alanine. (0.750 Molar.)*

pH (1)	$\frac{b-a}{c}$ (2)	b' (3)	Titration.				$\alpha$ (8)	[M] (9)	Rotation.				Deviation of pG'. (14)
			$\gamma_1$ (4)	$\gamma_2$ (5)	pG' (6)	pG' (7)			$\gamma_1$ (10)	$\gamma_2$ (11)	pG' (12)	pG' (13)	
0.57	-1.300	-0.869					4.54	15.1 <sub>3</sub>					
0.63	-1.250	-0.861					4.57	15.2 <sub>3</sub>					
1.18	-1.000	-0.912					4.37	14.5 <sub>7</sub>					
1.93	-0.750	-0.735	0.088		(2.20)		3.51	11.7 <sub>6</sub>	0.04 <sub>7</sub>		(2.48)		+0.09
2.39	-0.500	-0.495	0.265		2.37		2.51	8.3 <sub>7</sub>	0.26 <sub>8</sub>		2.37		-0.02
2.39	-0.500	-0.495	0.505		2.39		2.54	8.4 <sub>7</sub>	0.52 <sub>2</sub>		2.35		-0.04
2.89	-0.250	-0.248	0.505		2.39		1.55	5.1 <sub>7</sub>	0.51 <sub>4</sub>		2.37		-0.02
5.83	0	0	0.752		2.40		0.63	2.1 <sub>6</sub>	0.76 <sub>7</sub>		2.37		-0.02
6.05	0	0					0.65	2.1 <sub>7</sub>					
9.32	0.250	0.250		0.250		9.80	0.78	2.6 <sub>6</sub>		(0.17 <sub>6</sub> )		(9.99)	+0.19
9.32	0.250	0.250		0.250		9.80	0.80	2.6 <sub>7</sub>		(0.20 <sub>3</sub> )		(9.91)	+0.11
9.79	0.500	0.500		0.500		9.79	1.02	3.4 <sub>6</sub>		0.47 <sub>6</sub>		9.83	+0.03
10.28	0.750	0.749		0.749		9.80	1.23	4.1 <sub>6</sub>		0.73 <sub>8</sub>		9.83	+0.03
11.63	1.000	0.989					1.43	4.7 <sub>7</sub>					
13.10	1.250	0.907					1.45	4.8 <sub>8</sub>					
Average.....			2.39				9.80						

A sample of anhydride equivalent to a 0.0500 M solution was weighed into a calibrated flask. 15 equivalents of alkali were added. The solution was diluted to 50.0 cc. and was then allowed to stand at room temperature for 2 hours. The rotation in a 4.00 dm. tube was  $-0.48^\circ$ , which is equivalent to a rotation of  $-0.19^\circ$  in a 4.00 dm. tube for a 0.0200 M solution.

Four 10.00 cc. samples of this mother solution were introduced into 25.0 cc. flasks. The calculated volumes of standard acid required to leave an excess of 0, 1.000, 3.00, and 5.00 equivalents of alkali respectively were added. The solutions were then diluted to 25.0 cc. and their rotations were measured. The experimental results are recorded in Table II.

The titration-rotation data for dextro-alanyl-dextro-alanine

TABLE II.

*Rotation of Hydrolyzed Dextro-Alanyl-Dextro-Alanine Anhydride.*

<i>a</i>	$\frac{b}{a}$	$\alpha$
0.0200	0	-0.19
0.0200	1.000	-0.18
0.0200	3.00	-0.18
0.0200	5.00	-0.19
0.0500	15.00	-0.48
0.0200	15.00	-0.19

anhydride were then determined by the following procedure. Individual samples equivalent to 0.0500 M solutions were weighed into 25.0 cc. flasks. The required volumes of standard alkali (or acid) were then added and the solutions were made up to volume. The rotations were measured as rapidly as possible and 10.00 cc. samples were taken at once for the measurement of the degree of hydrolysis by the method described in the following paragraph. The pH values of the original solutions were determined on the 15 cc. residues (Table III).

To determine the degree of hydrolysis in each of the above experiments, the 10.00 cc. samples of the original solutions were introduced into 25.0 cc. flasks. The quantities of standard acid (or alkali) calculated for exact neutralization were then added



and the solutions were made up to 25.0 cc. The rotations are given in Column 6 of Table III.

*Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine*.—The material used in these experiments gave the following analysis.

No. 624.

Calculated for  $C_{10}H_{18}O_5N_4$ . C 43.76, H 6.64, N 20.44.

Found. " 44.10, " 6.83, " 20.26 (Kjeldahl).

The following two series of experiments were run.

*Series A*.—A solution of 0.200 M tetrapeptide was prepared. Samples of 2.50 cc. plus the calculated volumes of standard acid

TABLE III.  
*Dextro-Alanyl-Dextro-Alanine Anhydride. (0.0500 Molar.)*

pH	$\frac{b-a}{c}$	$b'$	$\alpha$	$[M_x]$	Neutralized solution.		Anhydride.		
					$\alpha$	$h$	$[M]$ (corrected).	$\gamma$	pG'
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
						per cent			
0.78	-4.00		-0.91	-45.5	-0.35		-45.5		
7.05	0		-0.93	-46.5	-0.37		-46.5		
11.94	0.250		-0.88	-44.0	-0.36		-44.0	0.00 <sub>2</sub>	13.6
12.20	0.500	0.042	-0.83	-41.5	-0.37		-41.5	0.04 <sub>3</sub>	13.6
12.37	0.800	0.090	-0.72	-36.0	-0.35		-36.0	0.09 <sub>1</sub>	13.4
12.48	1.000	0.086	-0.74	-37.0	-0.37		-37.0	0.08 <sub>3</sub>	13.5
12.75	2.00		-0.57	-28.5	-0.33	2 <sub>2</sub>	-30.0	0.14 <sub>4</sub>	13.5
12.93	3.00		-0.47	-23.5	-0.29	4 <sub>4</sub>	-23.6	0.19 <sub>9</sub>	13.5
13.01	4.00		-0.34	-17.0	-0.28	5 <sub>0</sub>	-10.8	0.31 <sub>0</sub>	13.4
13.12	5.00		-0.27	-13.5	-0.32	2 <sub>8</sub>	-9.7	0.32 <sub>0</sub>	13.4
13.25	7.00		-0.15	-7.5	-0.30	3 <sub>9</sub>	+2.6	0.42 <sub>7</sub>	13.4
	15.00		+0.07	+3.5	-0.36		+3.5	0.43 <sub>5</sub>	
Average.....									13.5

(or alkali) were made up to 5.00 cc. with water. The rotations were measured in 2.00 dm. tubes. The results are recorded in Table V.

In the experiments above pH 7 the polariscope readings were difficult to make because of the formation of a small quantity of flocculent material upon the addition of standard alkali.

*Series B.*—Individual samples of the tetrapeptide equivalent to a 0.1000 M solution were weighed into flasks. The calculated volumes of standard acid (or alkali) were added and the solutions were made up to 25.0 cc. with water. These solutions were then centrifugalized to free them from the flocculent precipitate which obscured the readings in the experiments of Series A. The polariscope readings were then taken in 4.00 dm. tubes and the concentrations of the solutions were determined by analysis. The corrections for the concentrations are given in Table IV and the data of the titration-rotation experiments in Table V, together with the data from Series A.

TABLE IV.

*Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.*  
*Experiment B.*

Correction of rotations to 0.1000 M concentration on the basis of total nitrogen determinations.

$\frac{b-a}{c}$ (1)	Total N in 1.00 cc. (2)	$\alpha$ (3)	$\alpha$ (corrected). (4)	[M] (5)	Amino N in 1.00 cc. (6)	$\frac{\text{Amino N.}}{\text{Total N.}}$ (7)
	mg.				mg.	
-0.500	5.39	13.54	13.94	348.5		
0 (Control.)	5.55				1.56	0.281
0.250	5.25	12.33	13.03	325.7		
0.750	5.46	12.18	12.37	309.2		
1.000	5.51	11.92	12.01	300.2	1.62	0.294
2.00	5.33	11.55	12.04	301.0	1.54	0.289
5.00	5.46	10.45	10.62	265.5	1.59	0.291

In some experiments of Series B, amino nitrogen determinations were made on the solutions. The constancy of the ratio of amino nitrogen to total nitrogen proved that no appreciable hydrolysis occurred, even in the solution containing 5 equivalents of alkali. The data are included in Table IV.

The values used in computing  $\gamma_1$  and  $\gamma_2$  from rotation data (Columns 11 and 12, Table V), are  $[M_1] = 362.5$ ,  $[M_0] = 331.0$ , and  $[M_2] = 300.6$ . Column 15 of Table V gives the deviations of  $pG'$  calculated on the basis of rotation data from the average values found by titration.

TABLE V.

Experi- ment.	pH	$\frac{b-a}{c}$	$b'$	Titration.				$\alpha$	[M]	Rotation.				Deviation of pG'.
				$\gamma_1$ (5)	$\gamma_2$ (6)	pG <sub>1</sub> ' (7)	pG <sub>2</sub> ' (8)			$\gamma_1$ (11)	$\gamma_2$ (12)	pG <sub>1</sub> ' (13)	pG <sub>2</sub> ' (14)	
A	1.28	-1.500	-0.900					7.25	362.5					
"	2.02	-1.000	-0.905					7.24	362.0					
B	3.30	-0.500	-0.495	0.505		3.31		13.94	348.5	0.45	3.39			+0.09
A	3.79	-0.250	-0.248	0.752		3.30		6.75	337.5	0.79	3.22			-0.08
"	4.82	0	0					6.63	331.5					
"	4.92	0	0					6.61	330.5					
B	7.47	0.250	0.250	0.250			7.95	13.03	325.7	0.17		8.16		+0.20
A	7.96	0.500	0.500	0.500			7.96	6.36	318.0	0.43		8.08		+0.12
B	8.45	0.750	0.750	0.750			7.97	12.37	309.2	0.72		8.04		+0.08
A	10.77	1.000	0.991					6.08	304.0					
B	11.18	1.000	0.976					12.01	300.2					
A	12.52	1.500	0.987					6.10	305.0					
B	12.83	2.00	0.852					12.04	301.0					
"	13.35	5.00	0.831					10.62	265.5					
Average .....						3.30	7.96							

## THE DECREASE IN SUGAR METABOLISM AND DESTRUCTION OF INSULIN BY ULTRA-VIOLET RADIATION.

BY W. E. BURGE AND GEORGE C. WICKWIRE.

(From the Department of Physiology, University of Illinois, Urbana.)

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A great deal of interest has been aroused of late in ultra-violet radiation in connection with rickets (1). There are two things, at least, that strike one on reading the literature on the subject. One is the indifference as to the source of the radiation, so far as wave-lengths and intensity are concerned, and the other the complete ignorance of the wave-lengths absorbed. The only thing that is observed is the effect. This, of course, is the most important thing; but for an intelligent study of the subject it seems to us a knowledge of the wave-lengths used and their intensity, as well as their absorption, must be taken into consideration, for it is only the radiant energy that is absorbed that can be effective.

The present investigation was begun to determine what effect, if any, ultra-violet radiation has on sugar metabolism. For this purpose fairly transparent animal cells (*Paramecia caudata*) were used. These organisms were grown in large quantities on an infusion made of lake water, pond-lily leaves, and horse manure. The most difficult part of this investigation was the growing of these organisms in sufficiently large quantities and in fairly pure cultures. The organisms were collected and washed free of debris with the use of a small centrifugalizing machine. The centrifugalizing tubes were calibrated in cc., so the organisms were measured as they were collected. 5 cc. of the organisms in 100 cc. of liquid were used in each of the experiments. Air was kept bubbling slowly through the liquid containing the organisms to insure an adequate supply of oxygen. The sugars used were dextrose, levulose, and galactose. Sugar determinations were made according to the method of Benedict. The source of the ultra-violet radiation was a Cooper Hewitt quartz mercury arc, operated

at 220 volts and 3.5 amperes. The following is the description of a typical experiment.

45 cc. of paramecia were collected as described above and introduced into 900 cc. of aerated lake water. While being thoroughly mixed by pouring from one vessel to another, the 900 cc. of liquid containing the organisms were divided into three batches of 300 cc. each. Into one batch 300 mg. of dextrose were introduced, and to the remaining two batches 300 mg. of levulose and galactose respectively were added. When the sugars were dissolved, each of the 300 cc. of paramecia-sugar preparations was divided into batches of 100 cc. each. One batch of 100 cc. of the dextrose, as well as the levulose and galactose preparations, was set aside for the controls. The remaining six batches were poured into glass dishes 35 cm. in diameter, and half of this number was placed 400 cm. from the light, and the other half 150 cm. away. Sugar determinations were made immediately and subsequently at intervals, as shown in Fig. 1, Columns 1, 2, and 3. It will be seen that the paramecia utilized all three sugars, and that dextrose and levulose were used more rapidly than galactose. In this respect, these organisms resemble the higher animals, for it is known that mammals use dextrose and levulose more rapidly than galactose (2). It will also be seen that ultra-violet radiation decreased the utilization of all three sugars, the greatest decrease being produced in the organisms closest to the light. We have carried out, during the past year, a great number of experiments similar to the preceding, and at different distances from the light, with essentially the same results as those given in Fig. 1. It should be mentioned that whatever water was lost by evaporation during the exposure was taken care of by the addition of an equal volume of water, and that the organisms were not killed at the end of 12 hours of exposure, either at the 150 cm. distance or at the 400 cm. The movements of the organisms at 150 cm. distance were slowed down considerably, but the organisms were still alive and active. Those at 400 cm. distance appeared as active as the controls, and in fact, so far as appearance under the microscope is concerned, they could not be distinguished from the controls. It should also be mentioned that air was kept bubbling slowly through the sugar solutions containing the organisms at all times to insure an adequate supply of oxygen. Control experiments

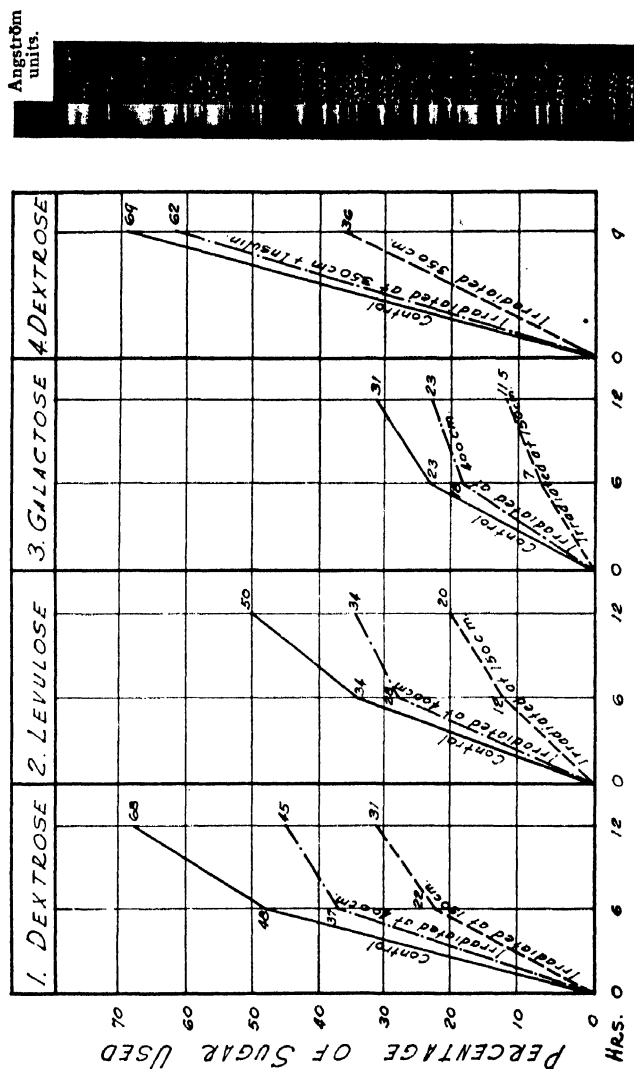


FIG. 1. In Columns 1, 2, and 3 are curves showing that animal cells (paramecia), utilize dextrose, levulose, and galactose and that ultra-violet radiation decreases this utilization. Column 4 shows that insulin prevents this decrease in sugar utilization by ultra-violet radiation. The photograph at the right of the chart shows the spectrum of the quartz mercury arc used in this investigation.

were carried out by bubbling air through sugar solutions not containing the organisms, to show that the bubbling of the air itself had little effect on the sugar content.

In Fig. 1 is also shown the photograph of the spectrum of the quartz mercury burner used. In another connection one of us had already made photographs of the spectrum of a burner very similar to the one used in this investigation, showing the absorption of ultra-violet rays by clear water, with and without paramecia. It was found, as has been known for a long time, that clear water absorbs a very small amount of the short wave-lengths. It was also found that when the paramecia were added to the water, none of the wave-lengths was completely absorbed, but the bands, particularly in the region of the short wave-lengths, were dimmed, thus showing that the organisms absorbed some of the radiation. In this connection the absorption by the cornea and the humors of the eye, as well as the short wave-lengths effective in coagulating protein and killing paramecia, was determined (3).

The next thing we were interested in was how does ultra-violet radiation decrease sugar metabolism. In connection with another piece of investigation we had already found that ultra-violet radiation destroys insulin. This suggested that the decrease in sugar utilization observed might be due to the destruction of insulin in the paramecia by the ultra-violet rays. To test out this idea, paramecia-sugar preparations were made similar to those already described. Insulin was introduced into some of the preparations exposed to the radiation and not into others. It was found that the organisms to which the insulin was added and exposed to the light utilized the sugar almost as rapidly as the controls, while the sugar utilization by the organisms to which insulin was not added and exposed to the ultra-violet radiation was much less than by the controls. The results of a typical experiment are given in Fig. 1, Column 4. The manner of adding the insulin was as follows: at the beginning of the experiment 2 cc. were added to the 100 cc. of liquid containing the organisms, after 4 hours 1 cc. was added, and at the end of 8 hours another 1 cc. added. By the addition of insulin we considered that the organisms would be kept supplied with this material, in spite of the destructive effect of the radiation on insulin. Since ultra-violet radiation destroys insulin and the addition of insulin in the manner described above keeps the sugar

utilization almost up to normal in the paramecia exposed to ultra-violet rays, it would seem a fair conclusion to draw that the decrease in the utilization of the sugar by the organisms exposed to the radiation was due to the destruction of the insulin in the cells by the radiation.

The following is proof that ultra-violet radiation destroys insulin. 5 units each of insulin were injected subcutaneously into medium sized rabbits, and convulsions were produced in  $1\frac{1}{2}$  to 2 hours. Dextrose was administered by a stomach tube, and the animals were relieved of the convulsions. Insulin was exposed to the radiation at a distance of 100 cm. for 9 hours. The injection of as much as 50 units of this irradiated insulin produced no convulsions in the rabbits, in which 5 units had previously produced convulsions.

#### SUMMARY.

1. *Paramecium caudatum* utilizes all the simple sugars: dextrose, levulose, and galactose. Dextrose and levulose are used more rapidly than galactose, just as is the case with the higher animals.

2. Ultra-violet radiation decreases the utilization of the sugars by these animal cells, without, at the same time, killing or injuring the cells.

3. Ultra-violet radiation destroys insulin.

4. The addition of insulin to the liquid containing the organisms prevents the decrease in sugar metabolism by the ultra-violet rays.

5. The decrease in sugar utilization brought about by ultra-violet radiation is attributed to the destruction of the insulin in the cells by the radiation.

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